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Rôle des communautés microbiennes dans la dégradation de la matière organique en forêt dans un contexte d'exportation intense de biomasse

Francois Maillard

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Thèse

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François Maillard

Rôle des communautés microbiennes dans la dégradation de la matière organique en forêt dans un contexte d'exportation intense de biomasse

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Préambule

LES ENJEUX ENVIRONNEMENTAUX ACTUELS

Facing global change: the millennium challenge for plant scientists

Texte collégial rédigé en aval du 41^{ième} Symposium New Phytologist 'Plant sciences for the future', à Nancy, France (avril 2018) et accepté pour publication dans la revue New phytologist.

Facing global change: the millennium challenge for plant scientists

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41st New Phytologist Symposium 'Plant sciences for the future', in Nancy, France, April 2018

Introduction

We entered the Anthropocene with the industrial revolution. This geological era is defined by the unprecedented impact of human activities on the planet's geochemical cycles, making of us the main driving force of Earth environmental changes (Crutzen, 2002; Steffen et al., 2011). Since the middle of the XXth century the human population tripled, reaching 7 billion today and probably 10 billion by 2050 (United Nations, 2015). This dramatic increase, associated to the improvement of the population welfare, led to overexploitation of natural resources. Intensive agriculture and industrialisation resulted in global warming, modification of nutrient cycles, pollution and reduction of wilderness; endangering the preservation of eco- and agro-systems (Steffen et al., 2011; Tilman et al., 2002; Ehrlich & Harte, 2015). Today, the challenge is not only to intensify agro-productions to feed, fuel and shelter the growing population; but to do so in spite of the consequences of climate change while lessening our impact on the supporting ecosystems (Godfray et al., 2010; Byrne et al., 2018; Ehrlich & Harte, 2015).

Plant sciences can play an important part in mitigating both the causes and consequences of the pressure population growth imposes to the environment. As the primary producer of eco- and agro-systems, plants are essential to assess and understand human driven environmental changes (Lin 2008; Loreau et al., 2001). They are also central tools to develop sustainable production methods (Godfray et al., 2010; Ehrlich & Harte, 2015; Byrne et al., 2018).

In this context, the 41st New Phytologist Symposium « Plant sciences for the future » was set as an experimental interdisciplinary platform. Bringing together early career and leader scientists from different fields of plant sciences, it aimed to promote the development of trans-disciplinary research projects to build a better understanding of the multiple aspects of the upcoming environmental challenges; and to produce robust solutions for society. A special debate chaired by Marc-André Selosse (Natural History Museum of Paris, France) and Richard Norby (Oak Ridge National Laboratory, USA) highlighted the critical topics and knowledge gaps the scientific community needs to fill in order to harness plant sciences to solve these societal issues. This event, held in Nancy, France on the 11-13th April 2018, hosted researchers of 70 universities, research institutes and companies representing 29 countries in the fields of Developmental biology, Evolutionary biology, Ecology, Plant microorganisms interaction, Physiology and Genetic engineering. In this article we outline how all plant sciences fields contribute to understanding the effects of global change and to developing innovative solutions to maintain agro-productions, promote sustainability and counteract climate change.

Exploring biogeochemical cycles

Human activities have altered global biogeochemical cycles. Colin Brownlee (Marine Biological Association, UK) illustrated the role of marine phytoplankton in the carbon (C) cycle, reminding that coccolithophores are responsible for much of the calcium carbonate formation on Earth. The increasing input of CO₂ into the atmosphere since the industrial revolution, responsible for ocean warming and acidification, is compromising the ability of coccolithophores to form calcium carbonate and therefore affecting the completion of the global C cycle (Orr et al., 2005). Brownlee demonstrated the role of proton channels in the calcification process of calcite coccoliths. Elucidating the cellular mechanisms involved in biomineralization is essential to minimize human impact on these critical species.

Forests represent a major C sink (Pan et al. 2011). Björn Lindahl (Swedish University of Agricultural Sciences, Sweden) highlighted the importance of plant-fungi interactions in nutrient cycling and soil fertility in boreal forests. Using high-throughput sequencing to elucidate boreal forest mycobiome and combining it to climatic, edaphic and forest productivity parameters, Lindahl's group showed that the composition of the fungal community is the principal driver of organic matter storage in those environments. Lindahl proposed that intensification of forest practices by changing soil fungal communities could improve the soil C stock in boreal forest but presents long-term soil fertility risks.

From the boreal forest to the steppe, Amy Austin (University of Buenos Aires, Argentina) demonstrated that photodegradation is a dominant force controlling C losses in semiarid ecosystems (Austin & Vivanco, 2006). Recent findings of her team suggest that photodegradation of the leaf litter promotes its subsequent biotic degradation by increasing accessibility of labile C compounds to microbes (Austin et al. 2016). Land-use or climate change altering vegetation cover could largely influence the effect of sunlight on C cycling in these ecosystems. Croplands are an anthropogenic biome that we could manage to increase potential C sequestration. CO₂ reaction with minerals naturally moderates atmospheric CO₂ and this effect has been enhanced since the emergence of land plants (Berner, 1997). David Beerling (University of Sheffield, UK) proposed to exploit this natural phenomenon by adding fast-reacting silicate rocks on croplands to trap CO₂. Eventually, weathering products could runoff in oceans and enhance alkalinity, counteracting acidification, and sustaining the growth of marine phytoplankton that we presented as crucial for the completion of C cycle earlier in this paragraph. Together, these results continue to highlight the importance of expanding our

knowledge about C and nutrients turnover on Earth to predict and actively minimize our impact on climate.

Assessing the effects of climate change on plant physiology

Global warming has increased intensity and frequency of extreme climatic events. High amplitude of temperature variation is the major cause of important plant losses in eco- and agro-systems (Boyer, 1982; Eiche et al., 1966; Hatfield & Prueger, 2015).

Plant pre-adaptation to climate variations could limit losses (Yordanov et al., 2000; Wikberg & Ögren, 2007). Drought acclimation of trees involves structural changes in wood formation and abscisic acid (ABA) is a key plant regulator of this acclimation (Gupta et al., 2017). Andrea Polle (University of Göttingen, Germany) showed the importance of ABA signal perception and response in wood formation of drought-stressed trees. Cecilia Brunetti (CNR, Italy) demonstrated how trees limit xylem conduits embolism by modulating their carbohydrate metabolism and how ABA is involved in restoring xylem transport ability.

Limiting water loss by modulating stomatal aperture is another plant survival response to drought. Predicting plant responses to different levels of drought is still difficult. Belinda Medlyn (University of Western Sydney, Australia) reviewed recent advances in « optimal stomatal theory » and presented a new in silico model to understand and predict stomatal responses to drought and heat.

Environmental stresses such as changes in temperature can affect plant metabolism and growth (Sampaio et al., 2016). Shuhua Yang (China Agricultural University, China) showed that stomatal conductance and, in consequence, leaf photosynthesis and respiration are affected by cold stress via the regulation of the CBF-dependent cold signalling pathway in *Arabidopsis* (Zhou et al., 2010). Owen Atkin (Australian National University, Australia) suggested that, by boosting plant respiratory metabolism, global warming could increase CO₂ release and influence the future atmospheric CO₂ concentrations.

Understanding plant physiologic and metabolic adaptive responses to climate change is a key factor for the production of efficient prediction models. These models are necessary to improve or develop novel management methods of eco- and agro-systems that could limit plant losses in the future.

Maintaining plant productivity

In our demographic context, maintaining population welfare depends on our ability to intensify agro-productions. Environmental changes are threats to the maintenance of crop yields in both agricultural and forests agro-systems. They have direct impacts on plant mortality and biomass (Lobell & Field, 2007; Schlenker & Roberts, 2008) and affect indirectly plant productivity by altering population dynamics of plant pests, symbiotic microorganisms and competitive species (Gregory et al., 2009; Lindner et al., 2009). Biotechnologic or agronomic solutions are necessary to lessen the consequences of global change on plant production.

In this frame, understanding the genetic basis of wood production in different tree lineages may help to mitigate the repercussions of abiotic stress on forest productivity through adapted management plans. Andrew T. Groover (UC Forest Service and UC Davis, USA) reviewed the genetic basis of evolution of woody plants and highlighted species-specific or conserved gene modules regulating the development of dicot and monocot cambium (Zinkgraf et al., 2017).

Environmental changes are modifying development and distribution of plant pests, threatening crop and forest productivity (Porter et al., 1991; Logan et al., 2003). Plant diseases are nowadays responsible for about 25% of crop losses (Martinelli et al., 2014). Controlling their outbreak is crucial to maintain plant productivity. A strategy to contrast future pest spreadings is to engineer crops resistant to a wide variety of pathogens. Ralph Panstruga's team (University of Aachen, Germany) explores the role of the MILDEW RESISTANCE LOCUS O genes (Jørgensen, 1992), encoding members of a family of membrane integral proteins conserved in plants, in conferring multiple resistances. They showed that mutations in MLO genes improved *Arabidopsis thaliana* resistance to some more leaf epidermal cell penetrating pathogens but increased susceptibility to microbes with different invasion strategies (Acevedo-Garcia et al., 2017). Stella Cesari (INRA, France), Tansley Medal winner, proposed to exploit the complex mechanistic and structural variability of nucleotide-binding domain and leucine-rich repeat-containing proteins (NLRs) to increase sensitivity or extend specificity of pathogen effector recognition (Cesari, 2017).

Understanding plants adaptive strategies to global change

Plants are increasingly exposed to new environmental stresses such as habitat degradation, climate change and the expanding range of invasive species and pests (Anderson et al., 2011). To predict the consequences of global change on ecosystems, it is necessary to understand the

different levels of plant adaptation (phenotypic plasticity, dispersion capacity and evolution) to new threats.

Plants can modulate the phenotypic plasticity of their neighbours by emission of volatile organic compounds (VOCs). André Kessler (Cornell University, USA) showed that VOCs emitted by *Solidago altissima* upon herbivore attack alter herbivore dispersal and feeding behavior through the modification of the metabolism of non-attacked plants. This indicates spreading the risk of herbivory to neighbours as a fitness-optimizing strategy. The high variability of VOCs types and levels in the field suggests the possibility of herbivore-driven natural selection on chemical communication (Morrell and Kessler, 2016). This might modulate crop adaptability to newly introduced pests.

Linda F. Delph (Indiana University, USA) reminded the audience that the phenotype is the direct interface between the organism and its environment and therefore at the center of evolution. She showed that genetic selection on key fitness traits such as flower number and height was strongly influenced by the environmental conditions in *Silene latifolia*. In-depth investigation of environmental factors influencing plant evolution may help predict phenotypic traits and fitness of plants in changing ecosystems.

Flower development is one of the most intricate and finely tuned processes influencing plant reproductive success. The floral organ must acquire specialized structures, bloom at the right time of the year and bear co-evolving traits with its pollinators. By taking advantage of the -omics technologies, several groups found that specific transcription factors (TFs) evolved to allow the formation of elaborate and diverse floral petals. Elena Kramer (Harvard University, USA) presented the role of the *AqJAGGED* gene, a TF involved in multiple key aspects in *Aquilegia* flower morphogenesis (Min & Kramer, 2017), while Hongzhi Kong (Institute of Botany, China) showed that *NpLMI1* and *NpYAB5-1* are involved in the control of *Nigella* petals shape.

Since the first observations of pollination systems by Darwin (1862), researchers are seeking evidence of pollinator-promoted selection for diverse floral shapes. Babu Ram Paudel (Yunnan University, China) showed how two alpine gingers (*Roscoea purpurea* and *R. tumjensis*) occur sympatrically and have similar morphology but are reproductively isolated through a combination of phenological displacement of flowers and different attracted pollinator. Global change might reshape these evolutionary boundaries and modify population or speciation dynamics.

Human impact on the environment will influence plant traits and drive their evolution by modulating plant fitness (resistance to pathogens, pollination, population dynamics). On the other hand, plant plasticity might provide a key for plant adaptability on the short term.

Innovative plant technology: a role for basic and applied science

Understanding the genetic and molecular basis of phenotypes is key to groundbreaking biotechnological applications: hence the importance of tight coordination and synergy between basic and applied sciences. The symposium hosted researchers interested in fundamental biologic mechanisms, scientists involved in both basic and applied research and developers employed in biotechnology companies aiming to bridge their complementary mindsets.

Understanding the molecular aspects of nutrient uptake and storage by plants is crucial to improve yield or nutritional properties of crops. By investigating the developmental biology of rooting systems in early land plants, Liam Dolan (University of Oxford, UK) showed that the development of rooting structures in land plants is tightly controlled by some conserved transcription factors networks (Proust et al., 2016; Breuninger et al., 2016). Such highly conserved key regulators can be used to enhance crops ability to access nutrients (Dolan et al., U.S. Patent Application No. 12/451,574). The fine tuning of lateral root emergence is another central aspect of root systems development. Keith Lindsey (Durham University, UK) showed how the 36-aa peptide POLARIS, orchestrating the auxin-ethylene crosstalk, modulates lateral root emergence (Chilley et al., 2006). These signalling mechanisms affect plants access to water and nutrients and mediate plant plasticity in a changing environment. The regulation of the level of reserves is also fundamental to plant nutrition. Alison Smith (John Innes Centre, UK) highlighted the importance of clock genes-modulated starch production and degradation for efficient plant sustainment (Graf et al., 2010; Scialdone et al., 2013). Arabidopsis leaves modulate the rate of starch degradation according to the duration of the night, in order not to starve before dawn (Fernandez et al., 2017). A better understanding of the dynamics of plant nutrient reserves may help engineering stress-resistant or nutrient-rich crops.

Examples of basic sciences translated into innovative plant technologies were given at the symposium. As presented above, David Beerling is exploiting silica weathering to counter accumulation of excess atmospheric CO₂. These results involved integrative studies spanning through geology, chemistry, economy and plant sciences, demonstrating once more the inestimable power of transdisciplinary research. Anne Osbourn (John Innes Centre, UK) showed that through co-expression, evolutionary co-occurrence and epigenomic co-regulation

genomes can be mined for biosynthetic gene clusters involved in production of secondary metabolites (Medema & Osbourn, 2016). Their genetic manipulation allows the production of specific chemicals at a lower-cost than conventional synthetic chemistry (Owen et al., 2017). Technical platforms and start-ups are being born in the exciting field of plant chemistry (Reed et al., 2017).

In conclusion, the symposium highlighted the need of integrative research to i) understand, model, predict the consequences of global change on ecosystems and plant physiology, productivity, epidemiology; ii) create innovative solutions to future challenges in the fields of food security, sustainable crop management and efficient production; iii) diffuse knowledge and know-how among specialists and general public. To this purpose, the symposium was closed by a public talk on plant-microorganisms interaction, given by Marc-André Selosse (Natural History Museum of Paris, France) on the beautiful background of the Hôtel de Ville of Nancy.

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INTRODUCTION GENERALE

INTRODUCTION GENERALE

1. Le rôle des microorganismes telluriques dans les écosystèmes forestiers

D'après la définition de l'Organisation des Nations Unies pour l'Alimentation et l'Agriculture (FAO) les forêts couvrent environ un tiers des terres émergées de la planète (FAO, 2015). Comme mentionné dans le préambule précédent, les écosystèmes forestiers sont des puits de carbone majeurs à l'échelle de la planète (Lal, 2005 ; Pan et al., 2011 ; Bar-On et al., 2018). La fertilité des sols est liée à leurs propriétés physiques (texture, agrégation, porosité etc.), chimiques (nutriments disponibles) et biologiques (édaphon) (Diacano and Montemurro, 2012). La fertilité des sols forestiers, qui présentent généralement des teneurs en nutriments disponibles faibles, dépend principalement du recyclage de la matière organique qui dérive des animaux, des plantes et des microorganismes morts (van der Heijden et al., 2008 ; Baskaran et al., 2017). La décomposition de la matière organique occupe ainsi un rôle central dans le cycle du carbone et des nutriments des écosystèmes forestiers (Schimel et al., 2012 ; Kyaschenko et al., 2017a). La matière organique est dégradée de manière abiotique par les radiations solaires (Austin et al., 2006 ; 2016) et biotique par la pédofaune et les microorganismes des sols (van der Heijden et al., 2008). La décomposition biotique est assurée dans les premiers stades par la macrofaune (termites, vers de terre etc.) et la mésofaune (collemboles, acariens etc.) qui participent au fractionnement et à l'enfouissement physique de la matière organique (Hättenschwiler et al., 2005 ; Pant et al., 2017). Les produits de fragmentation sont ensuite minéralisés par les microorganismes, principalement les champignons et les bactéries, via la production d'enzymes extracellulaires (de Boer et al., 2005 ; Štursová et al., 2012 ; Brabcová et al., 2016 ; López-Mondéjar et al., 2016 ; Tláškal et al., 201 ; Lladó et al., 2017). Les éléments inorganiques ainsi libérés sont disponibles pour les microorganismes et les plantes et ils contribuent à la formation de biomasse vivante. Les molécules carbonées issues de la décomposition sont métabolisées par les microorganismes hétérotrophes. Les champignons saprotrophes en raison de leur potentiel lignocellulolytique important peuvent être considérés comme les spécialistes de la dégradation de la matière organique végétale dans les écosystèmes forestiers (de Boer et al., 2005 ; Kohler et al., 2015 ; Talbot et al., 2015). Les communautés fongiques saprotrophes associées à la dégradation des litières et du bois sont bien décrites par les approches de séquençage d'amplicons haut-débit (Rajala et al., 2011 ; Voříšková and Baldrian, 2013 ; Hiscox et al., 2015 ; Hoppe et al., 2016 ; Purahong et al., 2016, 2017, 2018 ; Song et al., 2017). Néanmoins, les champignons saprotrophes participant à la décomposition de

la matière organique sous-terrainne comme les biomasses racinaires et microbiennes mortes sont relativement moins étudiés (Li et al., 2015 ; Fernandez et al., 2018). Les champignons ectomycorhiziens, en interaction symbiotique avec la majorité des ligneux des forêts boréales et tempérées sont, à l’instar des champignons saprotrophes, très abondants dans les sols forestiers (Buée et al., 2009 ; Schmidt et al., 2013). Mais contrairement aux champignons saprotrophes, leurs activités de dégradation de la matière organique végétale semblent très limitées (Kohler et al., 2015). Néanmoins, des résultats physiologiques et écologiques récents, principalement en contexte boréale, démontrent que certains genres fongiques ectomycorhiziens sont capables de dégrader la matière organique des sols (Rineau et al., 2012, 2016 ; Shah et al., 2016 ; Kyaschenko et al., 2017b ; Op De Beeck et al., 2018). Ces résultats physiologiques semblent cohérents avec des résultats écologiques démontrant la présence parfois abondante de champignons ectomycorhiziens dans le bois en fin de décomposition (Rajala et al., 2015 ; Mäkipää et al., 2017). Les champignons ectomycorhiziens sont également relativement abondants dans la nécromasse fongique, et ce du début à la fin de la décomposition (Fernandez and Kennedy, 2018). Les champignons ectomycorhiziens pourraient ainsi contribuer activement à la décomposition de la nécromasse fongique et de ses composantes comme la chitine. Ainsi, Lindahl &

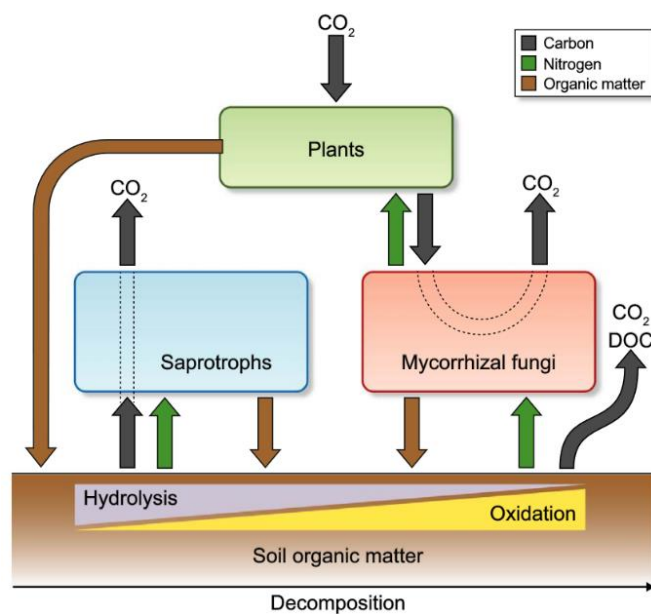


Figure 1. Flux hypothétiques de carbone et d’azote en forêt d’après Lindahl & Tunlid (2015)

Tunlid (2015) ont proposé un schéma hypothétique des flux de carbone et d’azote entre le sol, la plante et les champignons saprotrophes et ectomycorhiziens (Figure 1). Ces deux auteurs proposent que les champignons ectomycorhiziens utiliseraient principalement des mécanismes oxydatifs afin de mobiliser et de transférer vers la plante hôte les nutriments piégés dans la matière organique. Néanmoins le transfert d’azote extrait de la matière organique vers la plante par les champignons ectomycorhiziens est non démontré (Pellitier and Zac, 2018). De manière corrélative, Kyaschenko et al. (2017a) ont mis en évidence le rôle des communautés fongiques saprotrophes et ectomycorhiziennes dans la fertilité des écosystèmes forestiers boréaux. De façon analogue, les mécanismes de stockage du carbone dans les sols des forêts boréales et

tempérées semblent dépendre de la balance entre champignons saprotrophes et ectomycorhiziens (Fernandez and Kennedy, 2016). Les champignons ectomycorhiziens en interagissant avec les champignons saprotrophes pourraient contribuer à ralentir la dégradation de la matière organique et ainsi augmenter les capacités de stockage de carbone des sols forestiers. Ces résultats peuvent paraître contradictoire avec les études mettant en évidence les capacités de dégradation de la matière organique des champignons ectomycorhiziens (Bödeker et al., 2014 ; Clemmensen et al., 2015). On peut par conséquent imaginer que la capacité de dégradation de la matière organique n'est pas présente chez toutes les espèces de champignons ectomycorhiziens. Ainsi une étude large des capacités de dégradation de la matière organique des champignons ectomycorhiziens pourrait permettre d'améliorer nos connaissances du cycle du carbone et des nutriments des écosystèmes forestiers. De manière plus générale, ces résultats démontrent le rôle crucial des communautés microbiennes dans la fertilité et les capacités de stockage du carbone des écosystèmes forestiers.

2. Les rémanents forestiers comme source d'énergie

Le passage des énergies dites fossiles vers des énergies renouvelables pourrait contribuer à limiter les émissions anthropiques de CO₂ (Références). En juin 2018, le parlement et le conseil Européen sont parvenus à un accord concernant la directive 2030 sur les énergies renouvelables (ec.europa.eu). Cette directive fixe à présent un objectif de 32% d'énergie renouvelables, contre 27% initialement, dans la part totale des énergies de l'Union Européenne à l'horizon 2030. En 2015, au sein de l'Union Européenne, le bois représentait 5,9% du total des énergies consommées et environ 45% du total des énergies renouvelables principalement sous forme de granulés de bois (ec.europa.eu). Afin de répondre aux directives européennes sur les énergies renouvelables 2020, et maintenant 2030, une augmentation nette de la production de granulés de bois et produits assimilés est observée depuis 2008 (Figure 2). Ces granulés peuvent être produits à partir des troncs de ligneux. Néanmoins, afin de soutenir la demande croissante en bois énergie nous constatons une augmentation de l'utilisation de rémanents (branches, houpiers, feuilles, souches, racines) et petits bois (diamètres < 7cm) habituellement laissés en forêt après la récolte des troncs (UNECE, 2008 ; Mantau et al., 2010).

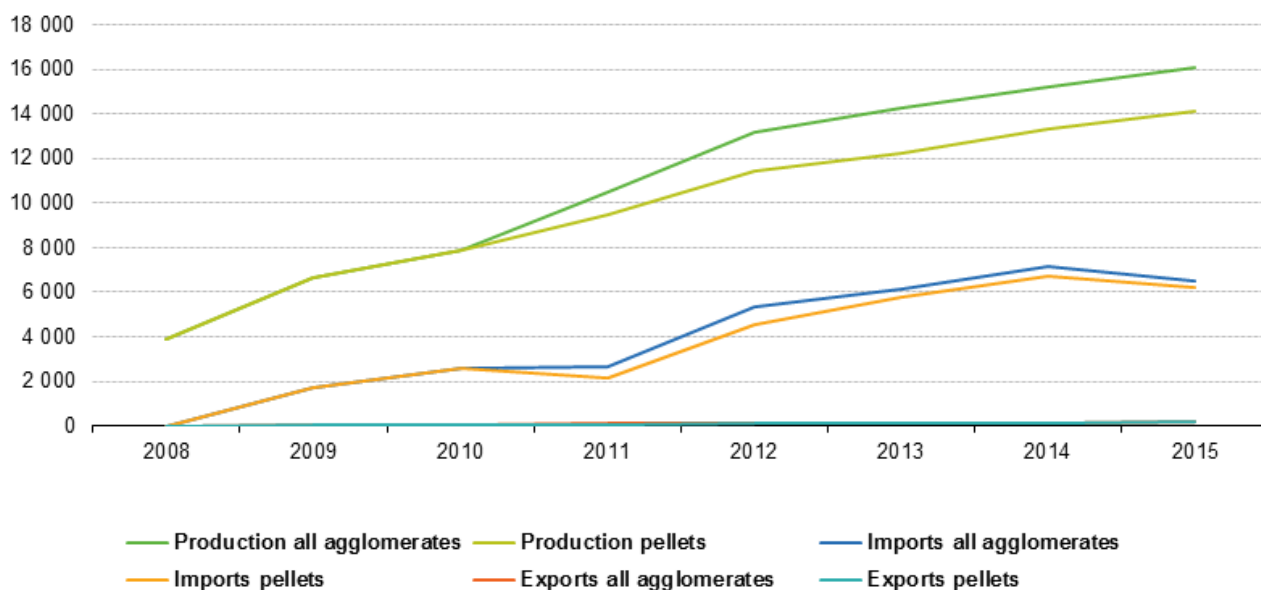


Figure 2. Production et échange de granulés de bois et d'agglomérés dans l'Union Européenne, EU-28, 2008–2015. (ec.europa.eu/eurostat/statistics-explained/index.php/Wood_as_a_source_of_energy)

Ainsi, d'après de nombreux auteurs, l'exploitation des rémanents forestiers comme source d'énergie pourrait représenter entre 25% et 100% des besoins d'augmentation des énergies renouvelables de l'Union européenne fixés par la directive 2020 sur les énergies renouvelables (Asikainen et al., 2008 ; UNECE, 2008; Anttila et al., 2009; Haberl et al. 2010; Bentsen & Felby, 2012). Il est démontré que des changements d'exploitation des forêts peuvent impacter de manière négative la capacité de ces écosystèmes à séquestrer du carbone au niveau des sols et des litières (Melin et al., 2010; Lindholm et al., 2011 ; Domke et al., 2012). De plus, par comparaison avec les troncs des arbres, les rémanents sont enrichis en nutriments souvent limitants dans les écosystèmes forestiers (Knust et al., 2016). Ainsi, la récolte de ces résidus d'exploitation, par rapport à la récolte des troncs uniquement, pourrait impacter la fertilité, la capacité de séquestration de carbone et les communautés microbiennes des écosystèmes forestiers.

3. Impact des pratiques sylvicoles intensives sur l'écosystème forestier

Les effets de la récolte des rémanents sur les propriétés des sols et la productivité des forêts ont été synthétisés dans plusieurs méta-analyses récentes. La publication d'Achat et al. (2015), démontre que les pratiques sylvicoles intensives, et plus particulièrement la récolte d'arbres entiers en comparaison avec la récolte des troncs uniquement, provoquent une diminution des stocks de carbone dans les sols des écosystèmes forestiers. Wall et al. (2012) et Achat et al. (2015) ont mis en évidence des effets négatifs des pratiques sylvicoles intensives sur les propriétés physico-chimiques des sols forestiers. L'exploitation d'arbres entiers en comparaison de la récolte de tiges uniquement conduit à une diminution des nutriments disponibles (N, P, K, Mg, Ca), une diminution du pH et une augmentation de la température et de la compaction des sols. Ces deux études montrent également que les pratiques sylvicoles intensives impactent de manière négative la croissance des arbres et plus particulièrement lorsque les branches encore feuillées sont récoltées. Néanmoins, une des principales limites de ces études et qu'elles concernent généralement l'évaluation des effets des pratiques sylvicoles intensives au cours d'une seule rotation forestière. En effet, une rotation forestière, séparant donc deux coupes, se déploie généralement sur plusieurs décennies et la majorité des études mesurent les conséquences des pratiques sylvicoles intensives seulement au cours des quelques années suivant une coupe définitive ou d'éclaircie. Les effets à long terme des pratiques sylvicoles intensives, sont par conséquent peu mesurés. Une des propositions faites pour évaluer les conséquences extrêmes des pratiques sylvicoles intensives sur les écosystèmes forestiers en dépassant les limitations temporelles consiste à mimer une diminution massive des flux de matière organique de l'arbre vers le sol (Stone, 2001). Ainsi, les feuilles/aiguilles et les branches sont des sources de matière organique qui peuvent être artificiellement exportées afin de contribuer de manière rapide à une diminution des apports de matière organique. Le travail de synthèse de Sayer (2006) même s'il n'a pas vocation à mimer les effets des pratiques sylvicoles intensives, démontre que le retrait de litière, a un impact très similaire sur l'écosystème forestier en comparaison des pratiques sylvicoles intensives. En effet, Sayer (2006) a mis en évidence une diminution des nutriments disponibles (N, P, K, Mg, Ca), une augmentation de la compaction et une diminution de la croissance des arbres en réponse à l'exportation des litières. L'exploitation de rémanents forestiers contribue de manière nette à une diminution de la productivité forestière. Ensemble, ces résultats démontrent que les pratiques sylvicoles intensives et notamment la récolte de rémanents diminuent les capacités de stockages de carbone et les nutriments disponibles des sols provoquant une diminution de la fertilité et de la

croissance des ligneux, et limitant par ailleurs le stockage de carbone dans la biomasse ligneuse vivante. Il reste important de noter que ces résultats sont extrêmement variables et pourraient dépendre de nombreux facteurs comme le contexte pédoclimatique ainsi que la composition en espèces végétales des sites étudiés. Les paramètres déterminants le gradient de réponse des paramètres physico-chimiques des sols et de la croissance des arbres mesurés en réponse aux pratiques sylvicoles intensives restent également à déterminer. En parallèle des approches expérimentales de terrain, les approches mathématiques de modélisation sont utilisées afin de prédire les effets des pratiques sylvicoles intensives sur le fonctionnement des écosystèmes forestiers. Les résultats des études de modélisation des effets des pratiques sylvicoles intensives sont contrastés voire même souvent opposés. Certaines études prédisent des effets globalement négatifs de la récolte des rémanents à court terme sur le stockage du carbone en forêt (Repo et al., 2014) alors que d'autres prédisent des effets positifs sur le cycle de carbone en forêt (Yemshanov et al., 2018). Ces résultats semblent montrer que la prédiction du fonctionnement des écosystèmes forestiers ne peut se limiter à une simple balance entre import et export de carbone et de nutriments mais résulte de l'interaction de toutes les composantes de l'écosystème et notamment des microorganismes non pris en compte dans les modèles jusqu'à présent. Les impacts des pratiques sylvicoles intensives réelles ou mimées sont relativement moins étudiés sur les micro-organismes telluriques des forêts que sur la productivité et la fertilité des sols forestiers. Mendham et al. (2002) a mis en évidence une diminution des biomasses microbiennes en réponse à la récolte d'arbres entiers en plantation d'Eucalyptus alors que Pérez-Battallon et al. (2001) n'a pas noté d'effet du retrait de litières et branches en forêt tempérée. Les fonctions enzymatiques de dégradation de la matière organiques diminuent en réponse aux retraits de rémanents (Achat et al., 2015). La composition des communautés bactériennes et fongiques change en réponse aux pratiques sylvicoles intensives alors que la diversité de ces communautés n'est que peu, voire pas du tout, impactée (Hartmann et al., 2008 ; 2012). Ces résultats indiquent que les compositions des communautés microbiennes sont sensibles à l'exploitation des rémanents en forêt. Néanmoins, que ce soit pour les communautés fongiques ou bactériennes, le lien entre statut taxonomique et fonctions dans l'écosystème est difficile à déterminer et les conclusions de ces études restent limitées. Les champignons ectomycorhiziens, relativement bien identifiés, font néanmoins figure d'exception. Wilhelm et al. (2017) a montré qu'en réponse à l'exploitation des rémanents forestiers l'abondance relative de la majorité des espèces ectomycorhiziennes diminue. Ces résultats sont soutenus par Mahmood et al. (1999) qui avait antérieurement montré une baisse du taux de mycorhization et de la densité des ectomycorhizes suite à la récolte de rémanents en peuplement d'épicéas. Ces

résultats sont surprenants car le lien entre diminution de la matière organique des sols et diminution de la diversité et de l'abondance des champignons ectomycorhiziens ne semble pas direct. Ces observations peuvent être liées à des effets indirects de l'exploitation des résidus comme des variations d'humidité, de température et de compaction des sols sur les communautés fongiques ectomycorhiziennes. De manière opposée, en forêt de Pin, plusieurs études révèlent que la manipulation de la matière organique a des impacts positifs sur la diversité et l'abondance des carpophores d'espèces de champignons ectomycorhiziens et sur le taux d'infection de plantules par les champignons ectomycorhiziens (Baar & Kuyper, 1993, 1998; Baar & De Vries, 1995 ; Baar & Ter Braak, 1996). La majorité des études portant sur l'effet de pratiques sylvicoles intensives sur la diversité et la composition des communautés microbiennes ont été conduites sur le réseau « long-term soil productivity » (LTSP) (Powers et al., 2005). Après coupe, différentes modalités d'exportation de résidus ont été mises en place et les études se tiennent entre 10 et 20 ans après la replantation. Ainsi, les écosystèmes forestiers étudiés sont relativement jeunes et les communautés microbiennes peuvent également être affectées par l'évènement de coupe récent qui est connu pour impacter fortement les communautés microbiennes (Kohout et al., 2018). Ainsi, si la diminution des activités enzymatiques de dégradation de la matière organique semble faire consensus, les effets de la récolte de résidus sur les communautés fongiques et plus spécifiquement les champignons ectomycorhiziens présentent des patrons de réponse opposés en fonction des études. Un schéma de synthèse des effets de la récolte de résidus proposé par Achat et al. (2015) permet de résumer cette partie (Figure 3).

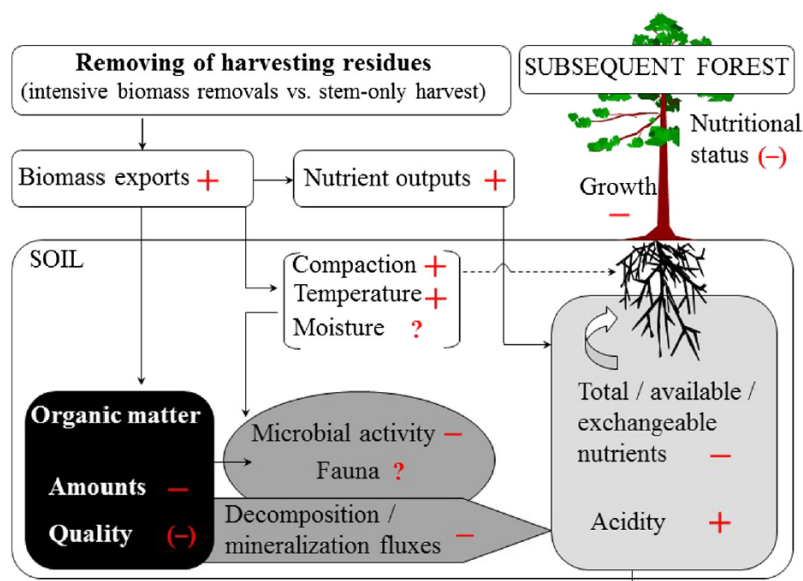


Figure 3. Impacts de la récolte de résidus sur l'écosystème forestier d'après Achat et al. (2015b)

Quelques lacunes nous apparaissent néanmoins concernant l'état des connaissances actuelles à propos des effets des pratiques sylvicoles intensives sur les écosystèmes forestiers. Si la diminution de la productivité forestière en réponse à la récolte de rémanents est démontrée dans plusieurs dizaines d'études, les effets sur la composition des communautés bactériennes et fongiques est limitée, tout comme les facteurs de résilience ou de sensibilité des écosystèmes forestiers à cette perturbation. Ensemble, l'acquisition de ces connaissances pourrait permettre (i) une meilleure compréhension mécanistique des effets des pratiques sylvicoles intensives sur les écosystèmes forestiers (ii) une identification des facteurs de sensibilité des forêts aux pratiques sylvicoles intensives (iii) le développement d'indicateurs biologiques de dysfonctionnement des écosystèmes forestiers en réponse aux retraits des rémanents.

4. Objectifs de la Thèse

1. Objectifs généraux

Nous nous proposons ici d'évaluer les effets de pratiques sylvicoles intensives réelles ou mimées sur la diversité taxonomique et fonctionnelle des communautés microbiennes. La mesure des effets d'un traitement sur un système biologique permet d'en déduire les conséquences mais non d'expliquer les mécanismes conduisant aux effets observés. Ainsi, nous proposons que la capacité de dégradation de la matière organique des champignons ectomycorhiziens est l'un des éléments qui pourrait permettre une meilleure compréhension du fonctionnement des écosystèmes forestiers et plus précisément des mécanismes de la réponse de ces écosystèmes aux pratiques sylvicoles intensives. Par conséquent, les capacités de dégradation de la nécromasse et de la chitine fongique par les champignons ectomycorhiziens sont ici étudiées.

2. Objectifs détaillés

Les objectifs détaillés de cette thèse sont :

- a. D'étudier l'effet de l'exportation artificielle de matière organique sur les fonctions microbiennes des sols en hêtraies de plaine.
- b. D'étudier l'effet de l'exportation artificielle de matière organique sur la diversité et la structure des communautés bactériennes et fongiques des sols en hêtraies de plaine.
- c. D'étudier les facteurs contribuant à la résilience des communautés microbiennes des sols à l'exportation artificielle de matière organique en forêt de hêtre.
- d. D'élargir nos observations, en étudiant l'effet de la récolte d'arbres entiers sur les fonctions microbiennes des sols en plantation d'eucalyptus.
- e. D'évaluer les capacités de mobilisation et de transfert de carbone et d'azote provenant de nécromasse fongique par le champignon ectomycorhizien *Paxillus involutus* en condition d'interaction symbiotique avec l'hôte végétale (*Pinus sylvestris*).
- f. De comparer les capacités de dégradation de la chitine au sein d'une collection de souches de champignons ectomycorhiziens.
- g. De quantifier les capacités de mobilisation d'azote provenant de chitine pour une collection de souches de champignons ectomycorhiziens et mycorhiziens à éricoïdes.
- h. D'étudier les liens entre les potentiels génomiques et les mesures fonctionnelles de dégradation de la chitine chez les champignons ectomycorhiziens.

5. Structure de la thèse

Cette thèse est composée de six chapitres pouvant être regroupés en deux parties distinctes. La première partie vise, par des approches *in situ*, à évaluer les potentiels effets des pratiques sylvicoles intensives sur le fonctionnement microbien des sols par des approches de retrait artificiels de matière organique (Chapitre I et II) ou correspondant à des pratiques sylvicoles réelles (Chapitre III). La seconde partie de cette thèse porte sur l'étude des capacités de dégradation de la matière organique *in vitro* des champignons ectomycorhiziens, en se focalisant sur une catégorie particulière de la matière organique : la nécromasse fongique (Chapitre IV) et la chitine (Chapitre V et VI).

La liste et le titre des chapitres qui composent la thèse sont les suivants :

CHAPITRE I: Soil microbial functions are affected by organic matter removal in temperate deciduous forest.

CHAPITRE II: Shift in soil bacterial and fungal community structures in response to organic matter removal in temperate deciduous forests.

CHAPITRE III: Enzymatic activities and community-level physiological profiles (CLPP) in subsoil layers are altered by harvest residue management practices in a tropical Eucalyptus grandis plantation.

CHAPITRE IV: First evidences that the ectomycorrhizal fungus *Paxillus involutus* mobilizes nitrogen and carbon from saprophytic fungus necromass

CHAPITRE V: N-Acetylglucosaminidase activity, a functional trait of chitin degradation, is regulated differentially within two orders of ectomycorrhizal fungi: Boletales and Agaricales.

CHAPITRE VI: Organic nitrogen mobilisation from fungal chitin was revealed by genomics and physiological evidences in a wide range of ectomycorrhizal fungi.

6. Méthodologie

Cette thèse inclus à la fois des expériences de terrains et des expériences conduites en conditions contrôlées et stériles.

3. Sites expérimentaux

Le réseau de placettes expérimentales « MOS »

L'effet d'une manipulation artificielle de matière organique sur les communautés microbiennes des sols et la physiologie de l'arbre en forêt tempérée est abordé dans les chapitres I et II. Ces deux chapitres se basent sur le dispositif expérimental de manipulation de la matière organique MOS (Matières Organiques des Sols). L'objectif principal de ce dispositif expérimental est d'étudier les effets de l'exportation de matière organique sur le cycle du carbone et des nutriments en forêt tempérée en lien avec la fertilité des sols et la productivité forestière (Akroume et al. 2016 ; 2017). Ce dispositif expérimental est composé de 18 placettes forestières incluant trois essences, le hêtre (*Fagus sylvatica L.*), le chêne (*Quercus robur L.* et *Quercus petraea Liebl.*) et le sapin de Douglas (*Pseudotsuga menziensis Mirb.*). Chaque placette expérimentale est divisée en quatre traitements :

1. **Référence (sylviculture française habituelle)**
2. Retrait des rémanents seulement
3. **Retrait des rémanents et retrait total de la litière chaque année**
4. Compensation du retrait des rémanents par apport de cendres.

L'installation et le suivi technique des placettes de hêtre et de chêne a débuté en 2014. Dans le cadre de ce travail de thèse nous nous sommes intéressés à la comparaison de deux traitements correspondant au retrait de matière organique le plus intense (traitement 3) en comparaison avec la sylviculture de référence (traitement 1). L'essence que nous avons étudiée est le hêtre (chapitres I et II) car pour des raisons pragmatiques, les six sites hêtres du réseau expérimental MOS sont ceux qui ont été mis en place les premiers et dont le retrait annuel de matière organique a été le mieux suivi.

Station expérimentale d'Itatinga

Le chapitre III portant sur l'étude des effets de pratiques sylvicoles intensives sur les fonctions microbiennes des sols en forêt tropicale a été réalisé sur une placette forestière du réseau CIFOR (Center for international forestry research) au Brésil. Ce site expérimental a pour objectif

d'étudier l'effet de la fertilisation et de la gestion des résidus (branches, cimes) sur les propriétés du sol et la productivité de plantations l'Eucalyptus. Dans le cadre du chapitre III nous avons comparé un traitement de récolte d'arbres entiers avec un traitement de récolte de tiges uniquement, considéré comme moins intensif car les résidus (branches, feuilles, cimes) ne sont pas exportés et laissés sur le sol pour décomposition.

4. Ressources biologiques

Les chapitres IV, V et VI concernent l'étude des capacités de dégradation de la matière organique microbienne (nécromasse fongique) des champignons ectomycorhiziens. Les souches fongiques étudiées appartiennent à la collection de l'INRA de Nancy (UMR 1136 IAM).

5. Ressources génomiques

Dans le chapitre VI nous comparons le potentiel génomique et fonctionnel de dégradation de la chitine chez les champignons ectomycorrhiziens et mycorhiziens à éricoïde. Nous avons bénéficié des ressources génomiques du Mycorrhizal Genome Initiative Consortium (MGI ; <https://genome.jgi.doe.gov/MGI/MGI.info.html>).

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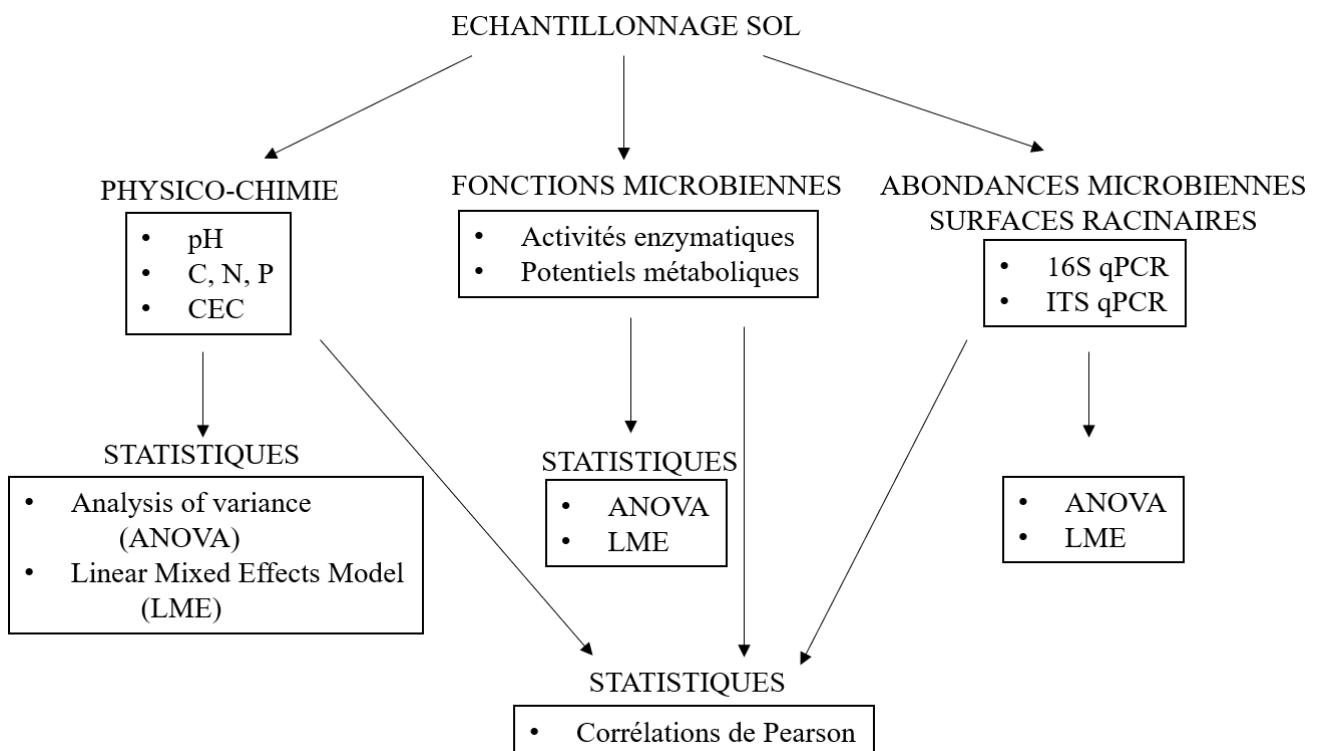
CHAPITRE I

Soil microbial functions are affected by organic matter removal in temperate deciduous forest

Article de recherche en révision dans la revue Soil Biology and Biochemistry.

INTRODUCTION AU CHAPITRE I

Le chapitre I concerne l'étude de l'effet de l'export de matière organique sur les fonctions des communautés microbiennes des sols en forêt tempérée. Cette étude se base sur le réseau MOS décrit précédemment. Le traitement de retrait intensif des rémanents et des litières a été comparé au traitement de référence sur les six placettes peuplées de hêtres du réseau MOS, à deux profondeurs de sol (0-5 et 5-10 cm). Les fonctions microbiennes ont été évaluées par des approches enzymatiques ainsi que leur capacité de métabolisation de substrats carbonés. Les biomasses microbiennes ont été déterminées par des approches de PCR (Polymerase Chain Reaction) quantitative de marqueurs moléculaires fongiques et bactériens (respectivement ITS et 16S). Les surfaces de racines fines ont également été estimées. Nous pouvons nous attendre à une diminution des teneurs en matière organique et nutriments des sols ainsi qu'à une diminution des abondances microbiennes en réponse à la manipulation de la matière organique. Les fonctions microbiennes de mobilisation de carbone et de nutriments à partir de matière organique devraient également diminuer en réponse à la manipulation de la matière organique.



Soil microbial functions are affected by organic matter removal in temperate deciduous forest

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Abstract

A growing demand for renewable carbon (C) has led to intensified forest management resulting in the use of forest residues (e.g. canopy, bark or litter layer) as energy sources with potential modifications of soil properties and tree productivity. Because microbes mediate the recycling of C and nutrients sequestered in organic matter, we investigated the effects of organic matter (OM) removal on soil properties, root surfaces, microbial functions and abundance using a new observational forest network. We compared leaf litter and logging residue removal plots to reference plots in six beech forests located in the northern half of France. After three consecutive years of OM removal, C and nitrogen (N) pools were not affected, but OM exportation decreased the cation exchange capacity and phosphorus (P) pool by respectively 12% and 30% in the topsoil (0-5 cm depth). Fine root surface area significantly increased by 21% in the subsoil (5-10 cm depth) in response to OM removal. Enzymatic activities involved in N and P mobilisation decreased from 12 to 38% with the manipulation of OM. Community-level profiling (CLPP) based on BIOLOG approach revealed that the metabolic potential of the microbial community strongly decreased in response to OM removal. Our results indicated that intensive forestry could affect microbial functions implicated in nutrients mobilisation. We demonstrated that soil organic matter (SOM) content is a predictor of microbial functions resistance to forest residue removal. We recommend that intensive forestry should be reduced or limited in beech forests characterised by lower soil OM contents.

Keywords

Organic matter removal, soil microbial functions, enzyme activity, CLPP, microbial biomass.

Highlights

- Soil microbial functions change in the short term in response to organic matter removal
- Organic matter removal leads to a decrease in the microbial metabolic potential and enzyme activities involved in nitrogen and phosphorus mobilisation
- The initial soil organic matter content is a predictor of microbial function sensitivity to organic matter removal

INTRODUCTION

Forests are major components of the carbon (C) cycle on Earth, sequestering large quantities of C (Lal, 2005; Pan et al., 2011; Bar-On et al., 2018). Consequently, managing forest C is a key challenge for mitigating climate change (Bonan, 2008; Canadell and Raupach, 2008). One of the proposed strategies to mitigate climate change consists of using more renewable energy sources in place of oil and coal. However, old-growth forests are defined as important C sinks, and this C, even soil C, could return to the atmosphere if these forests are disturbed (Luysaert et al., 2008; Bellassen and Luysaert, 2014). Furthermore, microorganisms participate in the dynamics of C and nutrient cycles in forests through processes that breakdown plant OM in a form that trees can assimilate (van der Heijden et al., 2008; Baskaran et al., 2017; Kvaschenko et al., 2017). Although this remains controversial in some countries, the application of forest management guidelines toward more intensive management practices is progressively observed (Achat et al., 2015a). Indeed, there is a demand to maximise post-harvest residue removal from forests and to divert this previously unused fresh plant organic matter (OM) into biofuel and energy production. This intensification of forest management practices (e.g. shorter rotation, stronger thinning, higher forest residue harvesting) may have negative impacts on soil properties and tree productivity as suggested by two recent meta-analyses (Achat et al., 2015a, 2015b). However, our understanding of how intensive forestry impacts soil microbial diversity and functions remains incomplete.

The effects of logging operations on soil microbial communities were mainly investigated in two different experimental networks in temperate and boreal forests. The Long-Term Soil Productivity (LTSP) study was established in 1989 to analyse the effects of OM manipulation

in a network of coniferous forests (Powers et al., 2005). Investigations of these LTSP sites demonstrated that bacterial and fungal community compositions changed in response to OM manipulation (Hartmann et al., 2009; Hartmann et al., 2012; Wilhelm et al., 2017). Additionally, Cardenas et al. (2015) used a metagenomic approach to reveal a decrease in the OM degradation gene potential in response to OM manipulation. The Detritus Input and Removal Treatments (DIRT) is an experimental network including eight sites in temperate deciduous forests (Nadelhoffer et al., 2004; Lajtha et al., 2005;). Kotroczo et al. (2014) and Veres et al. (2015) reported that the soil enzymes involved in organic C, N and P decreased in response to OM removal. The Kotroczo et al. (2014) and Veres et al. (2015) studies were focused at one site of the DIRT network. Other studies conducted independently of these two experimental networks highlighted the impact of OM removal on soil microbial functions (Hassett and Zak, 2005; Busse et al., 2006; Tan et al., 2008) and were also focused at one or two sites. Moreover, Achat et al. (2015b) indicated that long term impact of intensive forestry remains poorly examined and notably on soil microbial compartment.

In the present study, we measured soil parameters and associated microbial functions in six temperate beech forest sites of a new long-term observational French network, comparing OM removal treatment and conventional forest management (Akroume et al., 2016). This large-scale investigation aims to mimic the effect of middle / long-term OM removal thanks artificial and annual exportation of woody debris and litter layer (Stone, 2001). In addition to measurements of soil chemical parameters and fine root surface areas, we monitored the soil enzymatic activities and the community level profiling (CLPP) of the microbial communities in the topsoil (0-5 cm) and subsoil (5-10 cm) layers. Additionally, bacterial and fungal abundances were also assessed using quantitative PCR (qPCR). After three years of OM removal, we hypothesised decline of microbial abundance and associated functions implicated in OM degradation depending of the initial edaphic parameters of each studied site.

MATERIALS AND METHODS

Study site, experimental design and soil sampling

The study was conducted in six OM manipulation sites belonging to the “Matières Organiques des Sols réseau” (MOS network) in France (Table 1). All the sites are dominated by *Fagus sylvatica*. Each site contained 12 plots (40 m × 40 m), which corresponded to four OM manipulation treatments repeated three times as described by Akroume et al. (2016). In this

study, we focused on two treatments: conventional French forest management (reference plot: REF) and yearly residue and litter removal (OM removal: OMR). The OMR treatment consisted of manually removing every late autumn all coarse woody debris and leaf litter using a power-driven sidewalk sweeper. When we collected the soil samples, the OM removal treatment had been applied for three years. In each forest site, we selected the pair plots that had the most similar soil characteristics before harvest residue removal, based on the soil data published by Akroume et al. (2016). In September 2016, we sampled on both sides of the boundary separating adjacent REF and OMR plots at each site nine soil cores (15 cm diameter) for each of the two treatments (n=108 soils cores) as shown in Figure 1. Sampling was conducted when the soil moisture was relatively elevated and within the period of high soil microbial activity (Boerner et al., 2005; Kang et al., 2009). The separation into the topsoil (0-5 cm) and lower layer (5-10 cm) was performed in the field for each biological replicate (n=216 soils sampled). In the laboratory, the soil samples were sieved (2 mm) and homogenised before the enzymatic analyses and CLPP assay. An aliquot of each soil sample was freeze-dried for DNA extraction. Composites of three successive sampling points per depth, per treatment and per site were also made (n=72 composites). These composite soils were used for the Biolog® Ecoplate (Biolog Inc., Hayward CA., USA) approach and soil properties analysis.

Soil properties and the fine root surface area

Soil analyses were carried out by the Laboratoire d'Analyse des Sols d'Arras (INRA, France) on the composite soil samples (n=72). The cation exchange capacity (CEC) was determined according to the cobalt hexamine method. The pH was determined by the water method using a soil/water ratio of 1:5 (w/v). Total soil organic C and N contents were measured using a CHN analyser, while the P content was determined according to Duchaufour and Bonneau (1959). Beech tree roots were collected in each soil sample at the sieving step, and root length and total root surface were measured with the WinRHIZO system according to manufacturer's manual (Regent Instruments, Québec, Canada) (Wang and Zhang, 2009). Fine root surface areas defined as diameters less than 2 mm (Liu et al., 2018) were expressed as a projected area per soil core.

Bacterial and fungal abundance

Total nucleic acids were extracted from 0.5 g of sieved soil (n=216 soil samples) using the FastDNA SPIN Kit for soil (MP Biomedicals, Solon, OH, USA) with the addition of 20 mg of Polyvinyl-polypyrrolidone (Sigma-Aldrich, France). The bacterial and fungal abundances were measured as defined by Cébron et al. (2008) and Chemidlin Prévost-Bouré et al. (2011). Amplification reactions were carried out on 1 μ L of 1:10 diluted template DNA, standard plasmids (10^9 to 10^3 gene copies/ μ L) or water (negative control), in a 20 μ L volume using iQ SYBR green Supermix (Bio-Rad). Amplification temperature profiles consisted of 5 min at 98°C, followed by four steps of 40 cycles, 30 s at 98°C, 30 s at the primer-specific annealing temperatures (56°C and 50°C for 16S and 18S rRNA, respectively) and 10 s at 80°C to dissociate the primer dimers and capture the fluorescence intensity of the SYBR green. At the end, a melting curve analysis was performed from 70°C to 95°C, with a temperature increase of 0.4°C. Data are expressed as numbers of 16S or 18S copies per g of dry soil.

Enzyme activities

The first approach we used to characterize microbial functions was a soil enzyme activities assessment. We chose to measure eight hydrolytic and oxidative extracellular enzymes: beta-glucosidase, cellobiohydrolase, xylosidase, glucuronidase, laccase, N-acetylglucosaminidase, L-leucine-aminopeptidase and acid phosphatase. Briefly, 2 g of fresh soil (n=216 soil samples) was weighed and added to 40 ml of acetate buffer at pH 5.0 (methylumbelliferone fluorochrome and laccase assay) and pH 8.0 (methylcoumarine fluorochrome) to give a final concentration of 1:20 w/v. Diluted solutions were incubated at 4°C for one night under agitation. We applied the incubation times and substrate concentrations defined by Pritsch et al. (2011). For the laccase test, a solution of 2 mM 2,9-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS) was prepared in sodium acetate buffer at pH 5. In each well of a clear flat-bottom 96-well plate (Sarstedt, Newton, NC, USA), 200 μ l of the diluted soil solution was added to 50 μ l of 2 mM ABTS solution. After incubation at 25°C the plate was centrifuged at 4,500 g for 5 minutes, and 100 μ l of the clear solution was transferred into a new, clear flat-bottom 96-well plate, and measurements were performed at 415 nm (Bio-Rad model 550). For the microplate assays with the methylumbelliferone or methylcoumarine fluorochromes, in each well of a clear flat-bottom 96-well plate (Sarstedt, Newton, NC, USA), 200 μ l of the diluted soil solution was added to 50 μ l of the substrate solutions. After incubation at 25°C, the plate was centrifuged at 4,500 g for 5 minutes, and 50 μ l of the clear solution was transferred into a black opaque 96-well microplate

(Perkin-Elmer, Life Sciences) containing 50 µl of stopping buffer (Tris 2.5 M pH 10–11) in each well. Measurements were carried out using a Victor3 microplate reader (Wallac Perkin-Elmer Life Sciences, Villebon-sur-Yvette, France) with an excitation wavelength of 360 nm and an emission wavelength of 450 nm. Control measurements were made for each enzyme assay. Enzymatic activity (EA) was expressed in pmol of substrates released per g of dry soil per min. All of the chemicals were purchased from Sigma-Aldrich (France).

CLPP approach

We used the Biolog® Ecoplate as the second approach to explore microbial functions. For this experiment, 5 g of composite soils (n=72 soil samples) was diluted in 45 ml of sterile distilled water; after a brief agitation, it was diluted again to 1:20 w/v; and 100 µl of the 1:20 soil solution was used to inoculate the Biolog® Ecoplate microplate. Then the plates were incubated at 25°C, and colour development was measured at 590 nm (Bio-Rad model 550) after 72 h of incubation. Metabolism of the different substrates in the wells resulted in the reduction of tetrazolium, which changed from colourless to purple formazan. The OD 590 nm of the reference well was subtracted from the other wells containing carbon sources. These corrected absorbances were used for the subsequent analysis. The substrates were divided into five classes: carbohydrates, carboxylic and acetic acids, amines, amino acids and polymers as defined by Fraç et al. (2012). We also calculated the substrate utilisation richness (Garland, 1997), which represents the number of carbon substrates metabolised (corrected absorbance values > 0.25).

Data analysis and statistics

Statistical analyses and data representations were performed using R software (R Core Team 2016). Three-way analyses of variance (ANOVA) were used to test the following effects: site, depth and treatment impacts on soil properties, root surface, bacterial and fungal abundance, enzyme activities and CLPP data. Then, we used a mixed linear model with the lme4 package (Bates et al., 2015) with site as a random factor to evaluate the impact of the treatment on soil properties, root surface, bacterial and fungal abundance, enzyme activities and CLPP data in the function of the two studied soil layers (0-5 and 5-10 cm). From the Vegan package (Oksanen et al., 2013), we calculated Euclidean dissimilarity indices between the enzymatic activities (n=8 enzymes), and the CLPP assay (n=31 substrates) measured in the OMR and REF treatments within each site for both soil layers (0-5 and 5-10 cm). The Corrplot package (Taiyun Wei and Viliam Simko., 2017) was used to identify and represent the Pearson correlation

between the soil properties of the REF plots and the impact of OM removal on the root surface (a log 2-fold change OMR/REF), bacterial and fungal abundance (a log 2-fold change OMR/REF), enzyme activities (the Euclidean dissimilarity index between OMR and REF) and CLPP results of the Euclidean dissimilarity between OMR and REF). The levels of significance obtained are indicated in each figure and table legend.

RESULTS

Plant organic matter removal effect on soil properties

The total soil organic N, total soil organic C, soil organic matter (SOM) and pH were not impacted by OM removal (Table 2). In contrast, OM removal significantly affected the other soil parameters measured in the 0-5 cm soil layer (Figure 2), leading to an average decrease of P concentration (-30%), CEC (-12%) and C/N (-8%) in the OMR plots compared with the REF treatments. Significant responses of these soil properties to OM manipulation were restricted to the top soil layer (0-5 cm). Decreases in CEC and C/N were observed for the six studied sites, while P decreases occurred in the following sites: Gaillefontaine, Saint-Quirin and Verrières-du-Grosbois (Supplementary Figure 1).

Plant organic matter removal effect on bacterial and fungal abundances and fine root surface areas

OM removal significantly impacted bacterial abundance (Table 3). Indeed, the bacterial abundances significantly increased (+20%) in the OM removal plots, notably in the 0-5-cm layer (Figure 2). Although fungal abundance was not significantly affected by OM removal, it tended to increase in the OM removal plots. In addition, the fine root surface areas increased significantly in response to OM removal by 21% in the 5-10 cm soil layer of the OM removal plots. This increase was measured at all six studied sites (Supplementary Figure 2). Although the effect was not significant, this increase in the fine root surface area was also observed in the topsoil (0-5 cm).

Organic matter removal effects on enzyme activities

Beta-glucosidase, cellobiohydrolase, laccase and xylosidase activities were not significantly affected by OM removal (Table 4). Glucuronidase, N-acetylglucosaminidase, leucine aminopeptidase and acid phosphatase were affected by OM removal with significant decreases of 40%, 38%, 28% and 14%, respectively, in the 0-5-cm soil layer (Figure 2). Leucine

aminopeptidase was the only enzyme significantly impacted by OM removal in the 5-10 cm soil layer, with a significant decrease of 29% in the OM removal plots.

Organic matter removal effects on microbial metabolic profiles

OM manipulation caused substantial shifts in the soil microbial metabolic profiles. The CLPP approach revealed that amines, amino acids, carboxylic acids and carbohydrates substrates were affected by OM removal (Table 5). OM removal also impacted the total metabolic capacities and substrate metabolic richness of the microbial community. OM manipulation induced a significant decrease in amine (-62%), amino acid (-22%), carboxylic and ketonic acid (-28%), carbohydrate (-39%) and polymer (-25%) metabolic capacities in the topsoil layer (0-5 cm). The carbohydrate and amine substrates classes also significantly decreased in the 5-10-cm soil layer (-30% and -55%). The total metabolic capacities and substrate metabolic richness significantly decreased by 32% and 24%, respectively, in the 0-5 cm soil layer.

Correlation between soil properties and tree and microbial factors

With the goal of identifying the possible drivers and regulators of microbial functions and associated site sensitivity to OM removal, we explored the relationships between the local soil properties and OM removal impacts on the root surface, microbial abundance and microbial functions. Pearson correlation analyses were conducted between the soil parameters of each reference plot and the OM removal impact on bacterial and fungal abundances, fine root surface areas, enzymatic activities and microbial metabolic capacities for the two soil layers studied (0-5 and 5-10 cm). Euclidean dissimilarity indices calculated on the basis of the CLPP results between the REF and OMR plots for each studied site were significantly negatively ($P \leq 0.05$, $R^2 = 0.65$) correlated with the SOM content of the REF plots (Figure 3). The Euclidean dissimilarity index calculated on the basis of the enzymatic activities results between the REF and OMR plots for each studied site were also significantly negatively ($P \leq 0.05$, $R^2 = 0.81$) correlated with the SOM content of the REF plots. No other significant correlation was highlighted.

DISCUSSION

After three years of experimental OM removal, no significant changes in the soil C pool were measured. It is however worth noting the slight trend of a C decrease, especially for the Gailfontaine and Ban Harol sites in the topsoil layer. These findings are close to those obtained in some other forest sites (Johnson and Curtis, 2001; Barthès et al. 2008). Indeed, it

has been shown that experimentally or due to intensive forestry, OM removal does not strongly impact soil organic C pool (Nadelhoffer et al., 2004; Sayer, 2006; Fekete et al., 2011; Lajtha et al., 2014). Slesak et al. (2017) reported a decrease in SOM twenty years after forest floor removal on sites belonging to the LTSP network. This result is in agreement with our observations, highlighting that the impact of OM removal on the C cycle could strongly depend on the site local parameters, particularly C reservoir. Indeed, soil respiration is partitioned in recent photosynthates, which are metabolized by roots and microorganisms (Recent-C), and C that is respired during microbial decomposition of SOM that resides in the deeper soil (Reservoir-C) for several years or longer (Gaudinski et al. 2010). This soil organic C has a very long residence time and the sensitivity of forest sites (response timing) could be directly linked to the C pool size. Nevertheless, our monitoring has been performed after three years of OM removal treatment, suggesting that the intensity of the response will increase with the period of this recurring treatment. In the same way, we did not measure the effect of OM removal on the N pools. Changes in the N soil pools in response to OM removal commonly follow a similar tendency to those observed for soil C pools (Lajtha et al., 2014; Slesak et al., 2017). A slight but significant decrease in the CEC in response to OM removal was measured in the topsoil layer. The impact of OM manipulation on the soil P content was reported in other OM manipulation experiments (Wilhelm et al., 2013; Vadeboncoeur et al., 2014; Slesak et al., 2017). Due to the low level of P pools in forest soil, the decrease in P concentration at all six sites may suggest future soil P limitation, which is a serious concern for tree productivity (Goswami et al., 2018).

The impact of OM removal in temperate forests on fine root surface areas remains poorly explored. The increase of fine root surface areas in the subsoil layer in the six studied sites seems to be a rapid reaction of beech trees to OM removal. Similarly, litter fall removal significantly increased fine root production by 14% in 20-year-old Masson pine (*Pinus resinosa*) plantations (Da-Lun et al. 2010). Because the fine roots of beech are partly located in the upper organic horizons and in the fragmented litter, this increase could result directly from a vertical shift of the roots following the removal of litter. Furthermore, this increase of fine root surface areas could result also from a decrease of soil fertility in response to the OM removal. Indeed, Persson and Ahlström (1990) reported that N fertilization in Sweden Scots pine stands had negative effects on the development of fine roots, resulting in a reduction in the amount of fine roots to about 50% of that in the control. These fertilisation effects on the fine root biomass in forests have been reported in other studies (Cleveland and Townsend, 2006; Phillips and Fahey, 2007). Conversely, fertility reduction due to OM removal (e.g. decrease in

CEC and P concentrations in our study) could lead to stimulation of root growth. However, this phenomenon could have important impacts on soil functioning. Increase of fine root surface areas could lead to an increase of C and nutrients derived from root exudates and dead roots in the organo-mineral layers of OM removal plots due to the growth and rapid turnover of this tree organ (Kuzyakov, 2010). We can also suggest that a more important root exudation in these soil compartments could provide a priming effect at midterm and an increase in the degradation of recalcitrant C in the subsoil layers in the OMR plots compared to the REF plots (Dijkstra et al., 2013; Wang et al., 2016). This increase in fine root surface areas could also contribute to a change in the microbial community and their associated functions, including the ectomycorrhizal fungi that express different functional profiles related to their ecological niches (Buée et al., 2007).

In opposition to our hypothesis, bacterial and fungal abundances tended to increase in response to OM removal in this study. Some authors observed a decrease in microbial biomass as a response to OM removal (Li et al., 2004; Feng et al., 2009; Leff et al., 2012), while Fisk and Fahey (2001) did not observe any changes after OM removal. Smolander et al. (2010), who studied wood exportation scenarios in a boreal forest, revealed a site-specific effect with either an increase or a decrease of soil microbial biomass. Phospholipid fatty acid content, another indicator of microbial biomass, also increased in response to whole-tree harvesting and forest floor removal compared to a control plot (Ponder and Tadros, 2002). Observed increase of bacterial and fungal abundance could be link to the measured increase of fine root surface area potentially increasing the root exudations well known as C energy source for microorganisms (Kaiser et al., 2015). We could also hypothesized that OM removal may change microbial community composition toward populations presenting a greater carbon use efficiency (Sinsabaugh et al., 2013). The difficulty of identifying a generalizable trend of microbial abundance in response to OM removal confirms the need to find other disturbance predictors, such as microbial functions.

After three years of OM removal, enzyme activities involved in the mineralization of cellulose and hemicellulose (i.e., beta-glucosidase, cellobiohydrolase, and xylosidase) were not significantly impacted by the treatment. Our results are not consistent with the observations made by Kotroczo et al. (2014) and Veres et al (2015), who report a decrease of beta-glucosidase activity in response to leaf litter removal in temperate forests. This difference may be explained by the fact that they measured OM removal effects after 6 and 10 years whereas

our sampling occurred just after 3 years of OM removal. Using an isotope labelling approach, Leung et al. (2016) revealed that OM removal has a small impact on the hemicellulolytic degradation capacity of the microbial community. However, we hypothesize that the enzymes involved in plant cell wall degradation and C mobilisation similarly will decrease at our experimental sites in the coming years. The enzymes involved in P (acid phosphatase) and N (leucine aminopeptidase and N-acetylglucosaminidase) mobilisation were negatively impacted by OM removal in the topsoil layer and in the subsoil layer for N mobilisation. In our experiment, decreased acid phosphatase activity was correlated with decreased organic P content in the soil. In contrast, this link was not confirmed for the enzymes involved in N mobilisation. Indeed, soil N concentration had not yet decreased in response to OM removal, but the enzymes involved in N degradation had sharply decreased. Here, the total N does not allow estimates of the labile N pool that could regulate specific organic N mobilisation enzymes, such as chitinases or proteases. Similar to our results, many studies reported the sensitivity of N and P microbial mobilisation capacities in response to OM removal using mineralisation and enzymatic approaches (Perez-Batallon et al., 2001; Hasset and Zak, 2005; Tan et al., 2008; Smolander et al., 2010). These variations in the activities of the enzymes that degrade SOM could be linked to shifts in decomposition rates, indicating a reduction in the decomposition process in response to OM removal. Moreover, plant litter is rich in secondary compounds as tannins, which would contribute to microbial enzyme inhibition, as an important mechanism controlling some forest ecosystem processes (Harisson, 1971; Kraus et al., 2003; Joannisse et al., 2007). Consequently, litter exportation could directly change tannins availability, and microbial enzyme regulation. In addition, tannins and phenolics in fresh and decomposed beech litter are much more concentrated than other tree species, such as oak, Scots pine and black cherry (Lorenz et al., 2004). Indeed, Adamczyk et al. (2017) showed that tannin-enzyme interaction led to decreases but also increases of enzymatic activity due to protein conformation changes or tannin-enzyme precipitates. Variations in the microbial composition and their associated functions in response to OM removal may be a further explanation (Hartmann et al., 2012; Hartmann et al., 2009). Here, we confirmed that soil enzyme activities are more sensitive indicators of changes in response to OM removal than soil parameters, as observed by Caldwell and Gri (1999) and Tan et al. (2008). The availability of soil nutrients depends on the microbial enzyme activities that provide balanced resources for both microorganisms and the trees. In our study, the relative increase in microbial abundance coupled with the decrease in N and P mobilisation enzymes in response to OM removal could

modify the balance of these elements between microbes and trees, and increase the nutritive stress of trees in the OM removal plots.

An analysis of the Biolog® data only for the carbon source utilisation pattern demonstrated that OM removal had a significant impact on the microbial functional diversity and overall metabolic activity. Similar to the enzyme activities, these changes are mainly marked by a decrease in metabolic capacity and metabolic richness in response to OM removal in the topsoil layer. Nevertheless, in contrast to the enzymatic approach, the CLPP results in response to OM manipulation are scarce in the literature. In a critical review, Preston-Mafham et al. (2002) explained that the reduction of substrate degradation due to Biolog® Ecoplate could result from the decreased bacterial inoculum size. Using qPCR, we did not observe a decrease in the bacterial abundance and thus rejected this hypothesis. Because nutrient-poor forest soils are mainly dominated by slow-growing oligotrophic bacteria (Jones et al., 2009; Jeanbille et al., 2016; Žifčáková et al., 2016), we suggest that the removal of OM could increase the proportion of these oligotrophic bacteria in the manipulated plots, thus decreasing the relative abundance of copiotrophic bacteria. As the CLPP approach based on Biolog® Ecoplate is mainly driven by copiotrophic bacteria (Lladó and Baldrian, 2017), we propose that the differences in microbial functional profiles between the REF and OMR plots resulted from a change in the ratio between oligotrophic and copiotrophic bacteria.

We identified the general trends of the soil properties, fine root surface areas, microbial abundances and microbial function patterns to OM removal across the six studied sites. Nevertheless, we also observed that some sites appeared to be more susceptible to this disturbance simulating intensive forestry. These results reveal that certain sites are more sensitive to OM removal than others. Similarly, it was observed that the OM removal effects on soil properties and tree productivity depend on the initial nutrient pool, including organic C and, as a result, the high fertility mitigating OM removal's negative impacts (Egnell and Leijon, 1999; Ponder and Tadros, 2002; Powers et al., 2005; O'Hehir and Nambiar, 2010; Smolander et al., 2010). Here, we revealed that soil microbial functions followed the same trends observed for soil properties and tree productivity as those reported in the literature. We showed that for both the microbial functional diversity characterisations (CLPP and enzyme activities), site sensitivity was predicted by the initial SOM content. Thus, sites presenting the more important changes in soil microbial functions, with several declines in enzymatic activities and metabolic capacities, are the sites with the lower initial SOM levels in the topsoil.

CONCLUSION

Our results demonstrate that soil C and N pools in temperate forests were resistant to intensive OM removal while organic P concentration and CEC decreased. However, OM removal leads to decreases in N and P mobilisation enzymes. The metabolic capacity of the microbial community also declines in response to OM removal. These declines of enzymatic activities and metabolic profiles are not explained by a decrease in microbial abundance. In addition, the initial SOM content in the topsoil layer is a predictor of the soil microbial functions' resistance in response to OM removal. We recommend that forest practices limit intensive OM harvests, particularly in forests characterised by lower soil OM contents to ensure the maintenance of the soil microbes' role in tree nutrition and sustainable forest productivity. Relevant knowledge should thus be rapidly produced and efficiently transferred to decision makers to tackle this main challenge that forestry will have to face. As microbial functions appear to be sensitive to OM removal, it would be useful to develop microbial soil-based indicators for sustainable forest management.

TABLES

TABLE 1: Description of organic matter manipulation experimental sites.

Forest	Code	Species	First manipulation	Sampling		Coordinate	GPS Lambert93		Elevation	Humus type	Soil texture	Soil types (WRB 2014)
				date	Years		dirN	dirE				
Verrière du Grosbois	VE	Beech	16/05/14	Sept. 2016	55	6683070.9	948588.4	585	Mesomull/oligomull	Silty clay loam	Dystric Luvic Cambisols	
Gaillefontaine	GA	Beech	17/10/14	Sept. 2016	40	6952055.8	602895.7	290	Eumull/mesomull	Silty-loam	Eutric Cambisols	
Darney	DA	Beech	25/01/14	Sept. 2016	35	6779652.2	936520.6	500	Mesomull/oligomull	Sandy clay loam	Dystric Cambisols	
Compiègne	CO	Beech	25/04/14	Sept. 2016	30	6914427.8	695219.1	185	Mesomull	Sandy-loam	Stagnic Luvisols	
Ban d'Harol	BA	Beech	20/06/14	Sept. 2016	35	6783156.6	938890.7	350	Mesomull	Loam	Stagnic Dystric Cambisols	
Saint-Quirin	SQ	Beech	27/09/14	Sept. 2016	35	6842790.239	996528.371	400	Mesomull/dysmull	Sandy-loam	Dystric Cambisols	

TABLE 2: Three-way ANOVA of the influence of site, depth and treatment and their interactions in the soil properties (n=3).

	N	C/N	C	CEC	SOM	pH	P
Site	61.729 ***	29.947 ***	39.239 ***	29.874 ***	39.221 ***	23.096 ***	15.427 ***
Treatment	0.003	9.875 **	3.165	11.964 **	3.154	0.630	6.110 *
Depth	227.509 ***	4.350 *	70.586 ***	41.123 ***	70.730 ***	1.359	4.897 *
Site x treatment	3.668 **	3.852 **	0.884	0.619	0.882	2.603 *	1.421
Site x depth	12.699 ***	3.114 *	5.640 ***	2.178	5.627 ***	2.155	0.506
Treatment x depth	1.308	0.007	1.113	2.339	1.096	0.359	0.450
Site x depth x treatment	0.965	1.184	1.044	0.400	1.051	0.140	0.145

Values in the table represent the F-ratios and the levels of significance (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$). Abbreviations: N, total nitrogen; C, organic carbon; SOM, soil organic matter; P, organic phosphorus.

TABLE 3: Three-way ANOVA of the influence of site, depth and treatment and their interactions in the bacterial and fungal biomass and fine root surfaces (n=9).

	18S (number of copies)	16S (number of copies)	Fine roots surfaces (projected area per soil core)
Site	15.689 ***	8.511 ***	2.560 *
Treatment	1.248	4.521 *	3.203
Depth	20.574 ***	75.774 ***	50.179 ***
Site x treatment	1.614	9.022 ***	1.063
Site x depth	1.940 *	5.591 ***	0.959
Treatment x depth	0.819	3.188	0.140
Site x depth x treatment	0.979	5.249 ***	1.208

Enzymatic activities were expressed in pmol of substrates released per g of dry soil per min. Values in the table represent the F-ratios and the levels of significance (*P≤0.05; **P≤0.01; ***P≤0.001).

TABLE 4: Three-way ANOVA of the influence of site, depth and treatment and their interactions on soil enzymatic activities (n=9).

	Cellulohydrolase (EA)	Beta-glucosidase (EA)	Xylosidase (EA)	Glucuronidase (EA)	Aminopeptidase (EA)	N-acetyl- glucosaminidase (EA)	Acid phosphatase (EA)	Laccase (EA)
Site	5.516 ***	28.568 ***	10.887 ***	3.639 **	16.427 ***	1.474	20.578 ***	26.825 ***
Treatment	1.754	0.841	0.812	5.002 *	15.023 ***	11.121 **	8.654 **	3.624
Depth	37.850 ***	74.828 ***	46.645 ***	21.159 ***	15.228 ***	37.531 ***	124.429 ***	13.227 ***
Site x treatment	2.153	3.209 **	3.923**	2.006	1.811	2.609 *	1.840	2.077
Site x depth	1.568	1.214	1.463	2.297 *	0.710	1.045	3.092 *	0.277
Treatment x depth	0.872	0.013	0.176	8.318 **	0.406	6.022 *	1.387	0.351
Site x depth x treatment	1.170	0.253	0.222	1.329	0.645	0.939	1.506	0.160

Values in the table represent the F-ratios and the levels of significance (*P≤0.05; **P≤0.01; ***P≤0.001).

TABLE 5: Three-way ANOVA of the influence of site, depth and treatment and their interactions, in the soil CLPP (n=3).

	Amines (OD 590)	Amino acids (OD 590)	Carboxylic acids (OD 590)	Carbohydrates (OD 590)	Polymers (OD 590)	Total (OD 590)	Richness (n° of substrates metabolized)
Site	1.701	1.301	5.616 ***	8.985 ***	0.638	6.908 ***	5.661 ***
Treatment	18.548 ***	4.975 *	9.806 **	35.138 ***	1.742	27.127 ***	18.511 ***
Depth	14.639 ***	16.475 ***	62.083 ***	99.180 ***	1.598	84.133 ***	62.694 ***
Site x treatment	1.005	0.203	0.396	0.242	0.503	0.376	1.112
Site x depth	0.510	0.610	0.128	1.579	0.160	0.562	0.602
Treatment x depth	3.824	1.039	2.515	8.757 ***	1.997	7.430 **	7.750 **
Site x depth x treatment	0.775	0.233	1.251	2.235 *	0.396	1.354	0.472

Values in the table represent the F-ratios and the levels of significance (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

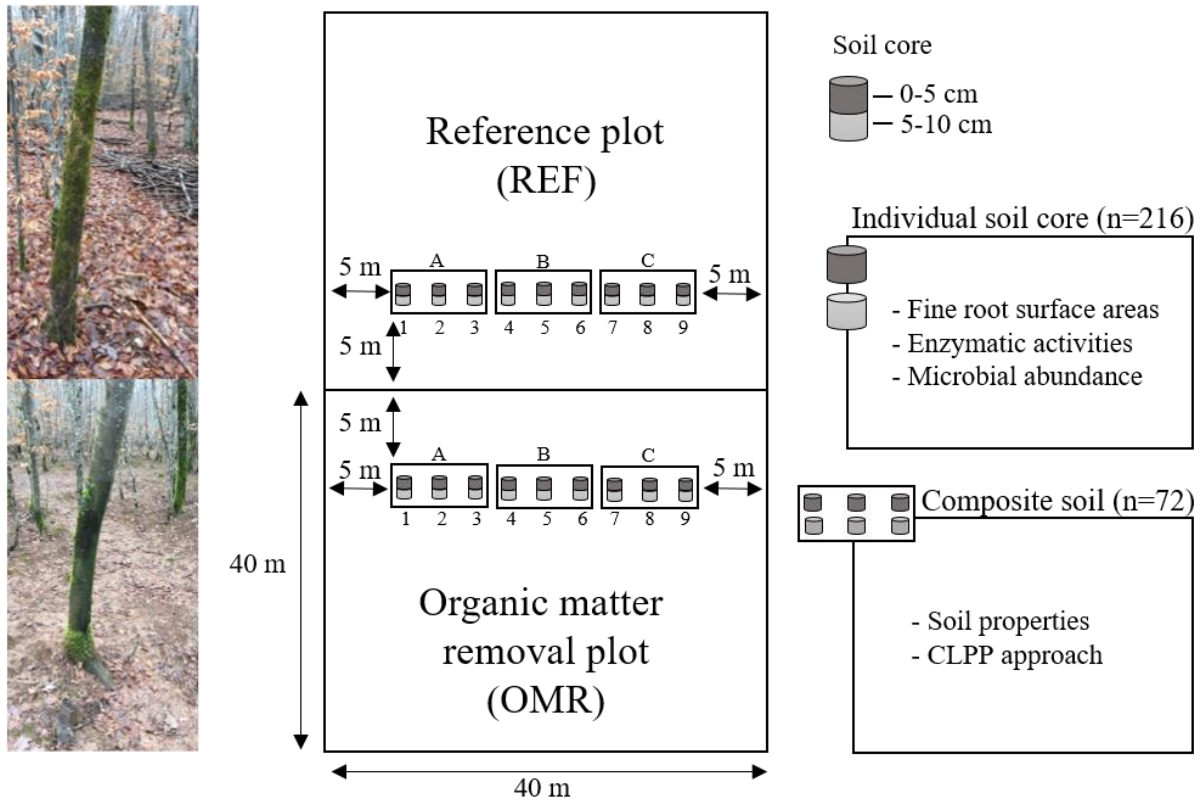


FIGURE 1. Schematic representation of the experimental design, sampling procedure and representative pictures of the REF and OMR plots of the Ban-Harol experimental site.

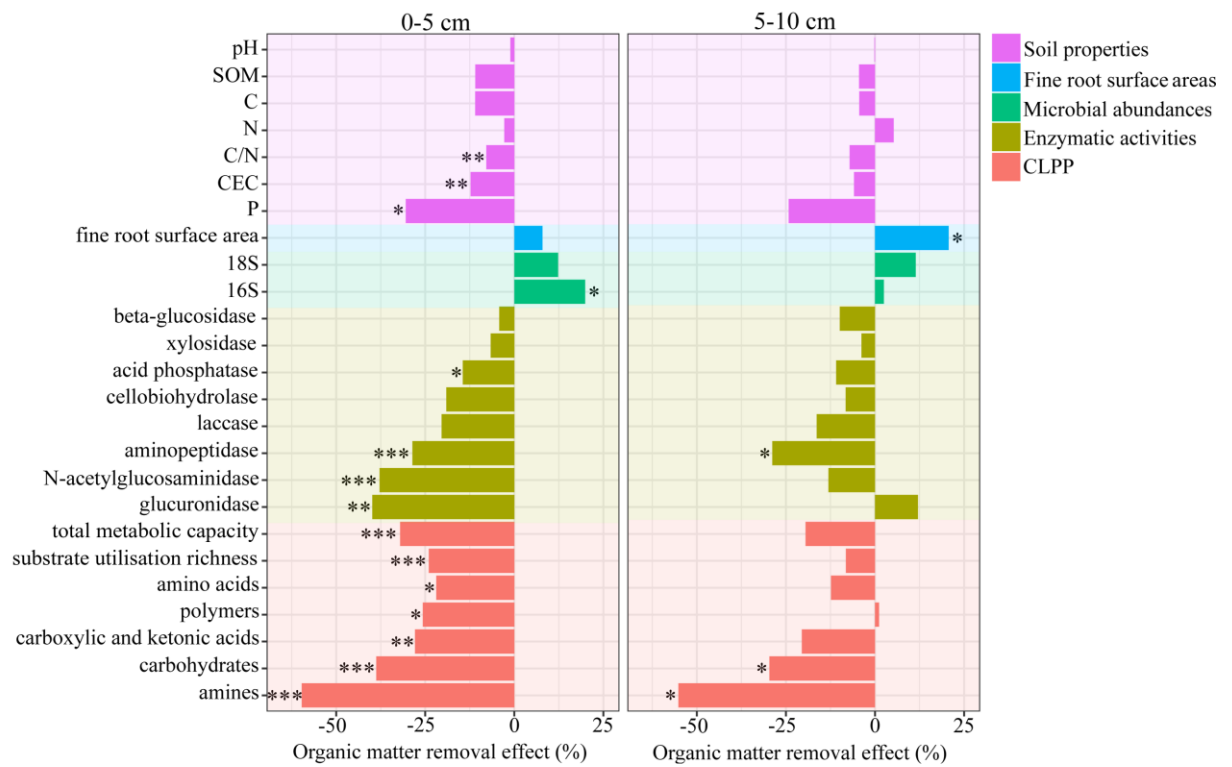


FIGURE 2. Differential analysis of the impact of OM removal (percentage change in the OMR plots calculated with REF plots as denominator) on soil parameters (pink), fine root surface areas (blue), microbial abundances (green), enzymatic activities (brown) and metabolic capacities (orange) of the soil microbial communities (in %) for the six studied sites in both soil layers (0-5 cm and 5-10 cm). Abbreviations: N, total nitrogen; C, organic carbon; SOM, soil organic matter; and P, organic phosphorus. Levels of significance are as follows: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

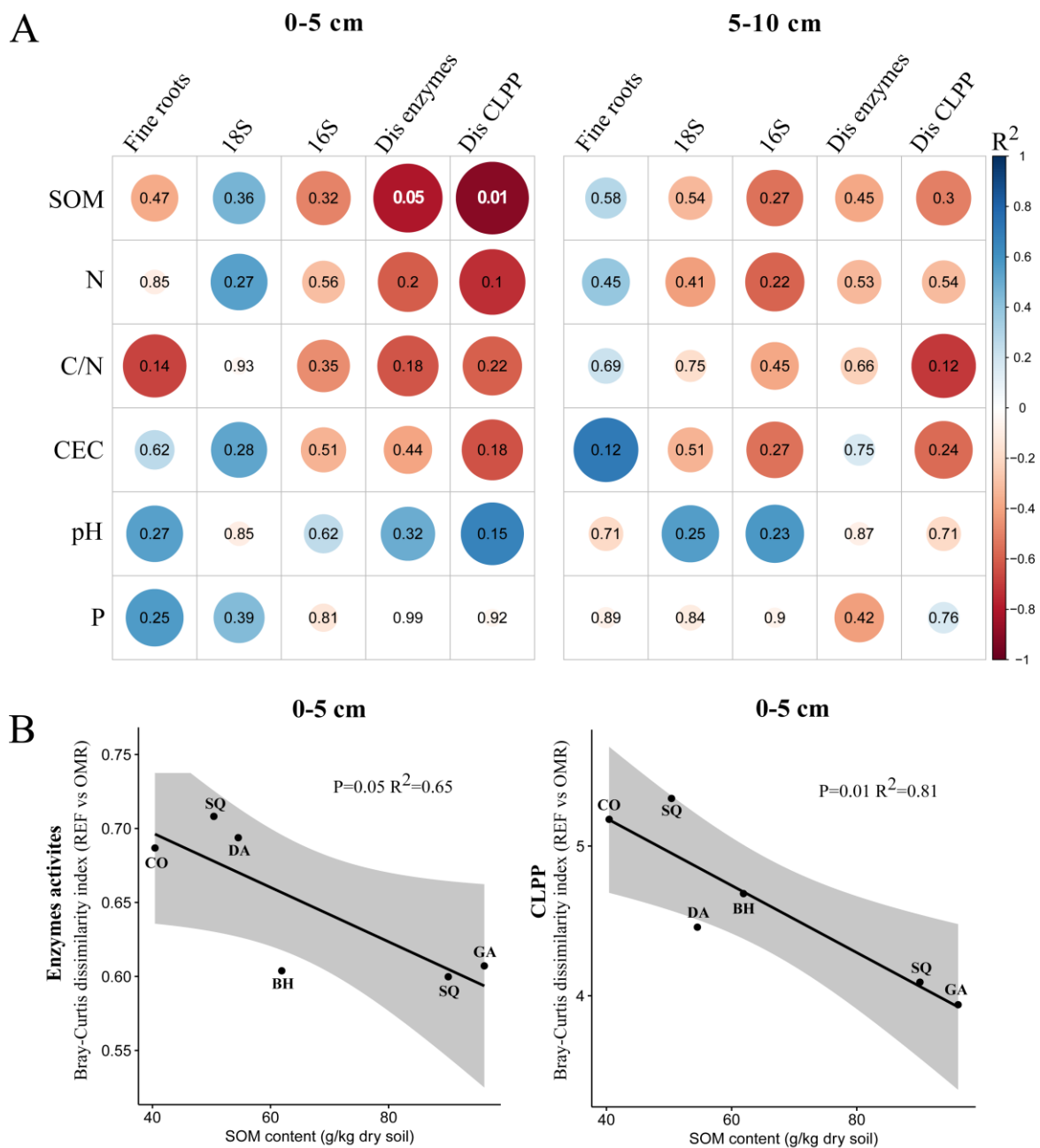
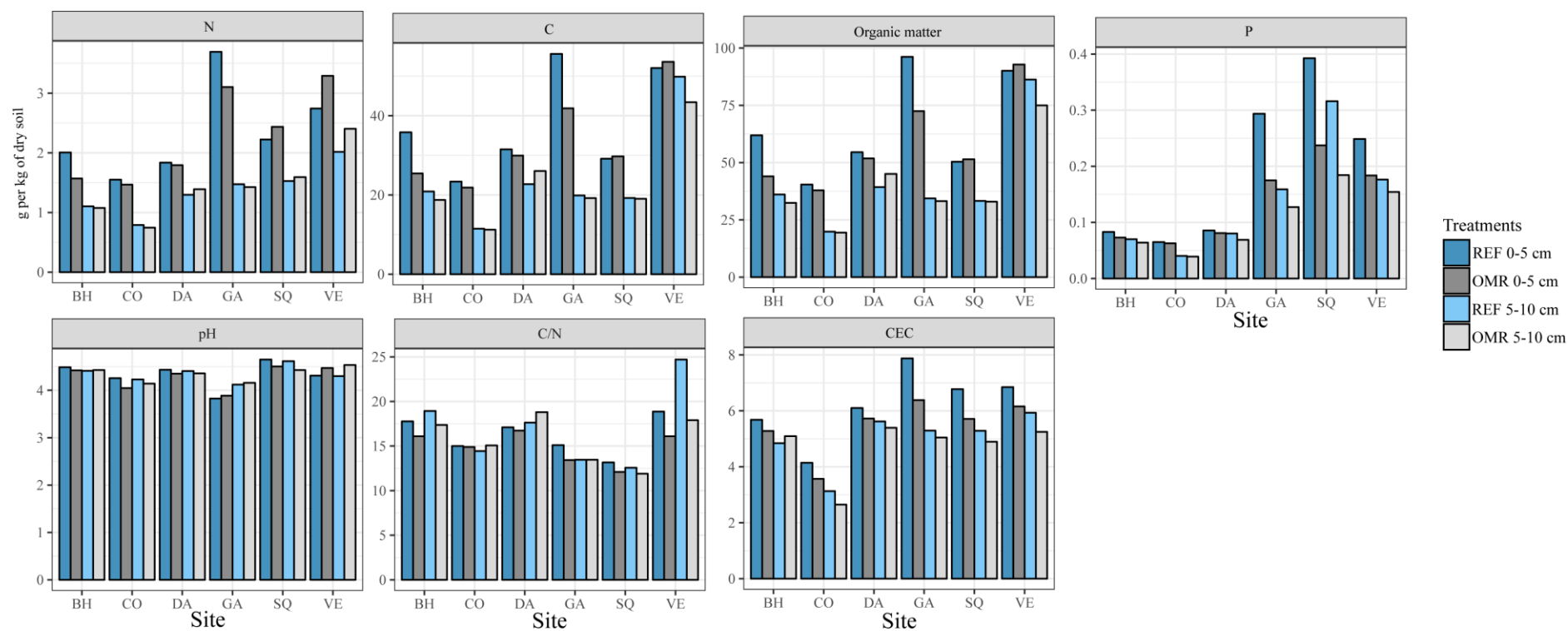
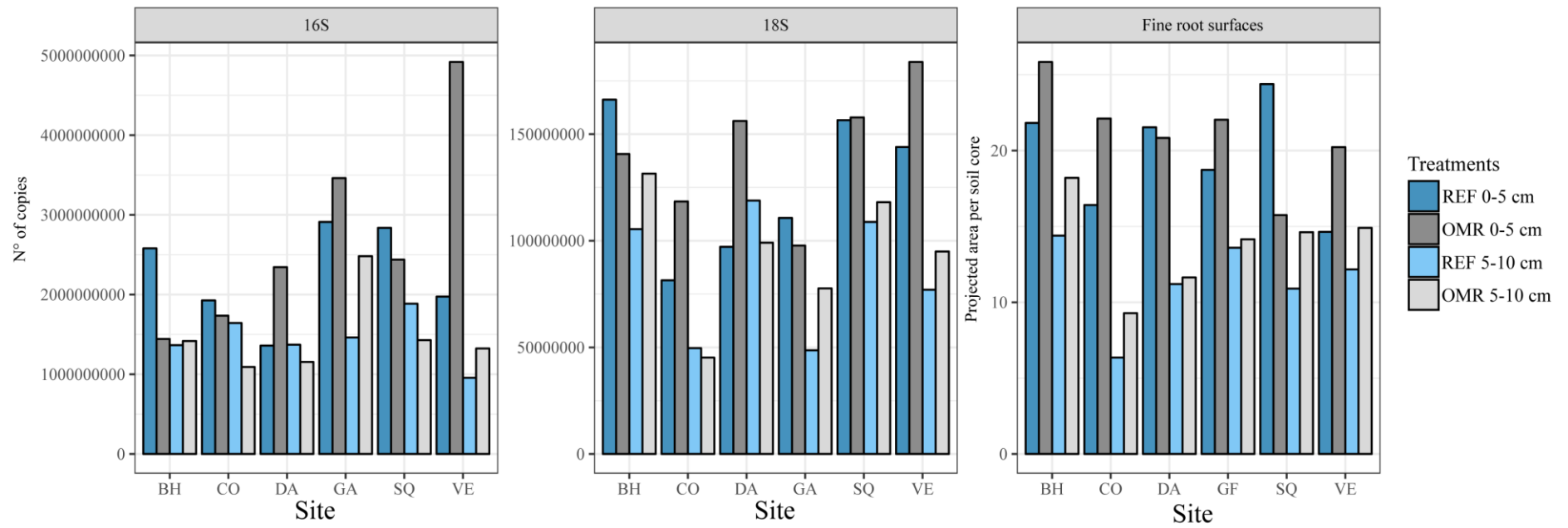


FIGURE 3. A. Pearson correlations between the soil properties of the REF plots and the following measurements: i) OM removal treatment on fine root surface areas, ii) bacterial and fungal abundance calculated for each site (log 2-fold change OMR/REF) and iii) dissimilarity index of enzymatic activities and the CLPP approach between the OMR and REF plots; for all sites ($n=6$) in both soil layers (0-5 cm and 5-10 cm). Colour of the circle depends on the direction of the correlation (blue = positive; red = negative). Size of the circle is proportional to the R^2 value. The value in the circle represents the P-value of the Pearson correlation. P-values in white are significant ($*P \leq 0.05$; $**P \leq 0.01$). Abbreviations: N, total nitrogen; SOM, soil organic matter; P, organic phosphorus; Dis enzyme: Euclidean dissimilarity index of enzyme activities calculated for each site; Dis CLPP, Euclidean dissimilarity index of the CLPP results calculated for each site. B. Representation of the Pearson correlation between the SOM content and Euclidean dissimilarity index of the enzyme activities and CLPP results calculated for each site for the 0-5 cm soil layer.

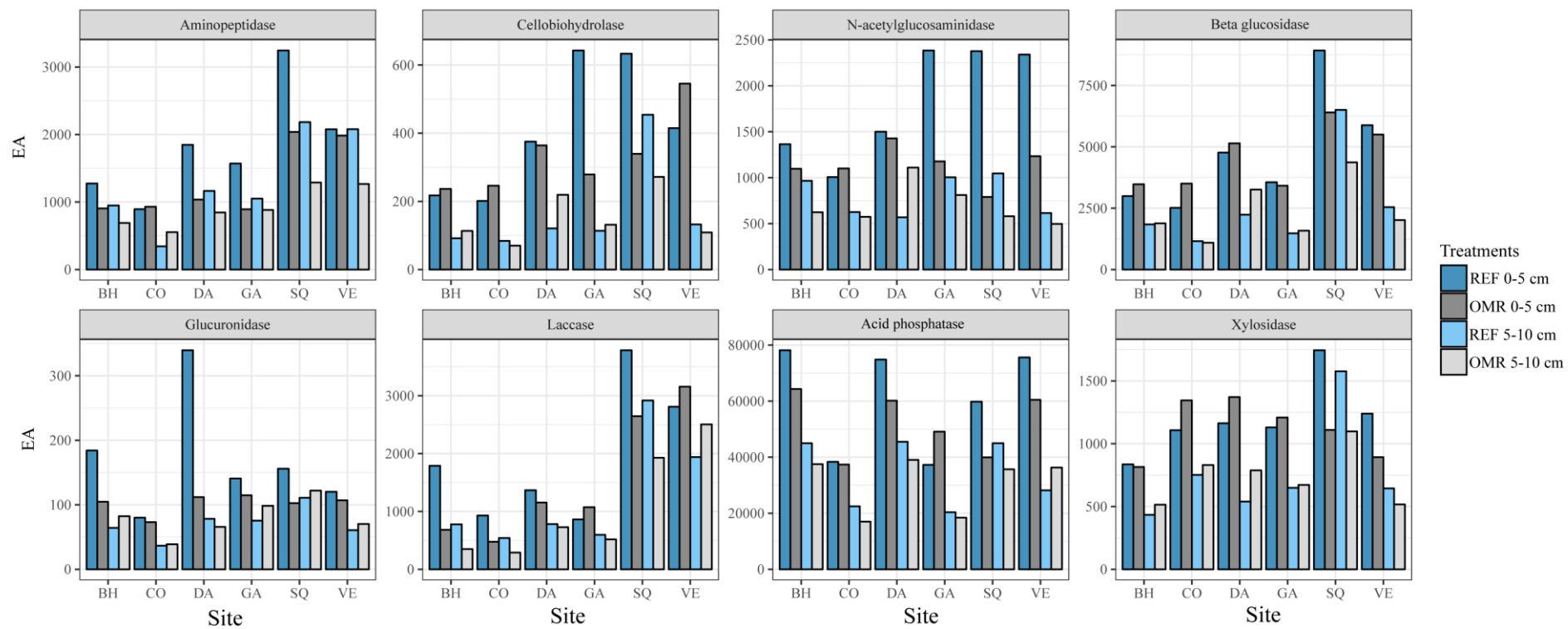
Supplementary data



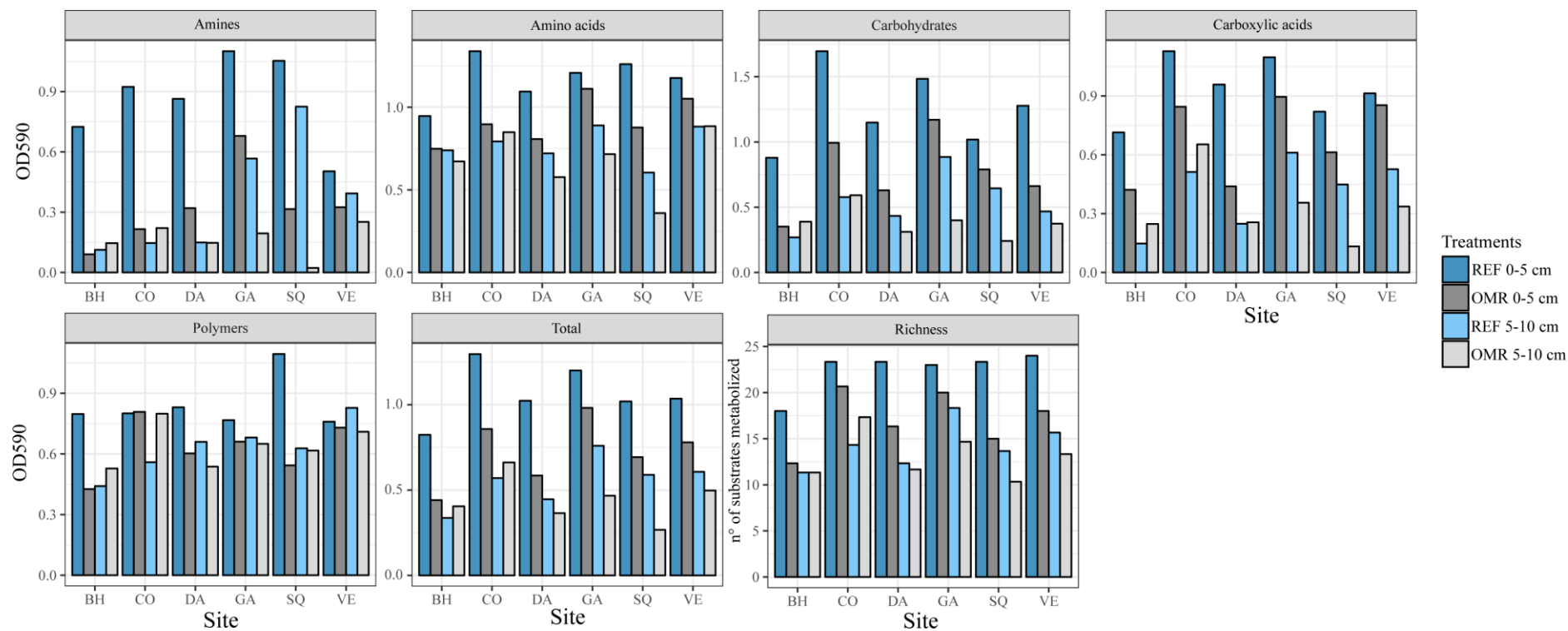
SUPPLEMENTARY FIGURE 1. Soil parameters in the different sites in the treatment function (OMR and REF) and depth (0-5 cm and 5-10 cm). Abbreviations: N, total nitrogen; C, organic carbon; SOM, soil organic matter; P, organic phosphorus.



SUPPLEMENTARY FIGURE 2. Bacterial and fungal abundances and fine root surfaces in the different sites in the treatment function (OMR and REF) and depth (0-5 cm and 5-10 cm).



SUPPLEMENTARY FIGURE 3. Soil enzyme activities in the different sites in the treatment function (OMR and REF) and depth (0-5 cm and 5-10 cm).



SUPPLEMENTARY FIGURE 4. CLPP results in the different sites in the treatment function (OMR and REF) and depth (0-5 cm and 5-10 cm).

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CHAPITRE II

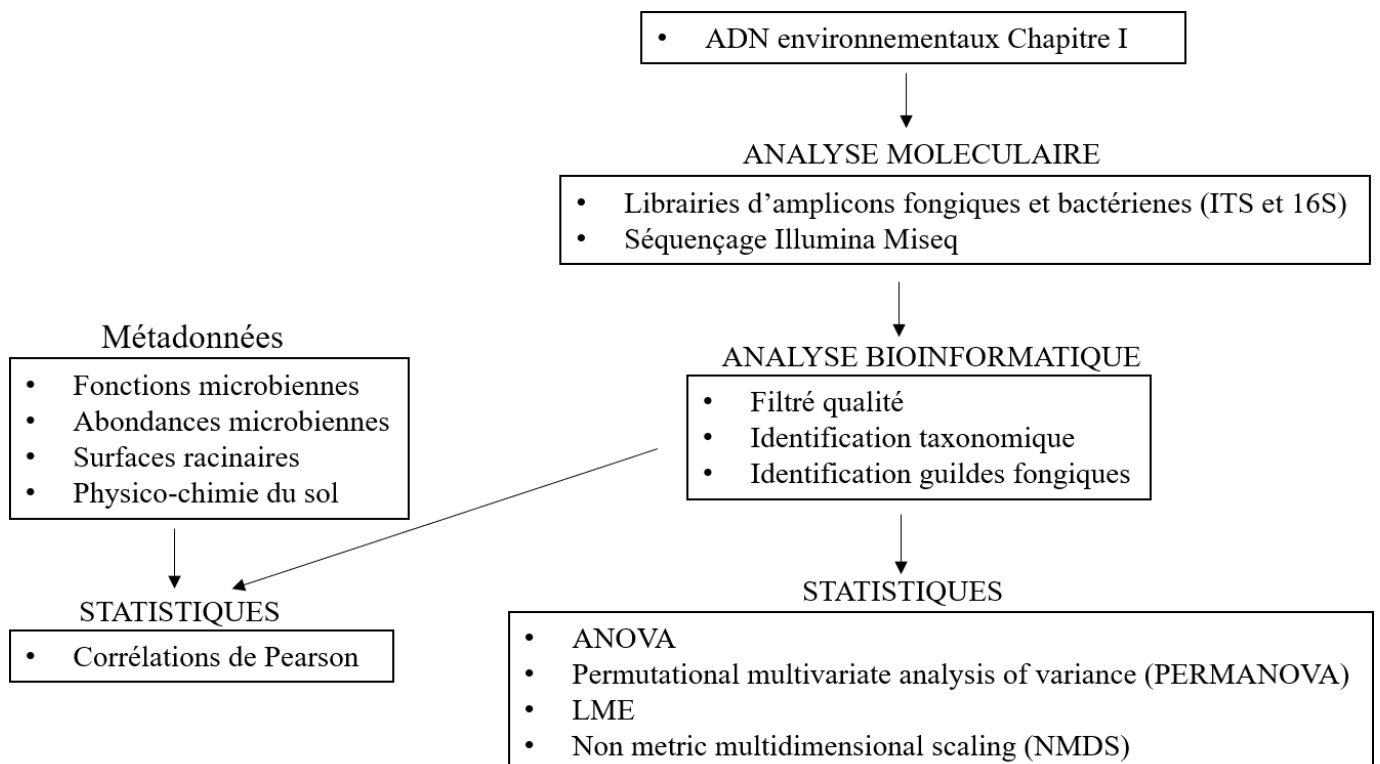
Shift in soil bacterial and fungal community structures in response to organic matter removal in temperate deciduous forests

*En Article de recherche en préparation en vue de soumission dans la revue
ISME Journal.*

INTRODUCTION AU CHAPITRE II

Nous avons vu dans le chapitre précédent que les fonctions de dégradation de la matière organique des communautés microbiennes changent en réponse au retrait de matière organique. On peut ainsi se demander si ce changement fonctionnel est associé ou non à un changement taxonomique des populations microbiennes.

Le chapitre II porte sur l'étude de l'effet de retrait de matière organique sur la diversité et la structure des communautés bactériennes et fongiques des sols en forêt tempérée. Cette étude se base sur le réseau MOS et les échantillons biologiques sont les mêmes que ceux du Chapitre I. A partir des ADN environnementaux utilisés pour la quantification des abondances fongiques et bactériennes nous avons réalisés des bibliothèques d'amplicons de marqueurs taxonomiques moléculaire fongiques et bactériens. Ces bibliothèques d'amplicons ont été séquencées avec la technologie Illumina Miseq. Après traitement bio-informatique afin d'assigner une information taxonomique à chaque séquence, nous avons comparé la diversité et la structure des communautés bactériennes et fongiques.



Shift in soil bacterial and fungal community structures in response to organic matter removal in temperate deciduous forests

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Abstract

The growing demand for renewable materials and energy is leading to intensified forest management practices. Consequently, combining high tree productivity and forest carbon storage with intensive forestry represents a major challenge. Microbial communities mainly drive soil processes responsible for soil carbon storage and soil fertility however we have poor knowledge about effect of intensive forestry on these populations. This study determined large scale impact of organic matter (OM) removal on soil bacterial and fungal communities across six OM manipulation sites across French temperate forests. Soil bacterial and fungal community compositions of reference (REF) and organic matter removal (OMR) plots were assessed using high-throughput sequencing. Analysis of 16S rRNA gene and ITS region libraries revealed that majority of bacterial and fungal taxa, including abundant groups, were affected by OM removal. After three years of OM removal, we measured a decline of copiotroph bacterial taxa associated with an expansion of oligotrophic bacterial taxa in the OM removal plots. Moreover, ectomycorrhizal and yeast growth form fungi relative abundance increased, and filamentous saprotroph fungi decreased in the OMR plots. This large scale molecular-based results demonstrated that OM removal induced major shift in forest soil bacterial and fungal communities. Moreover, we revealed that soil microbial communities were sensitive indicators of OM removal and are potentially useful for the monitoring of intensive forest practices impact on forest ecosystems.

Key words: organic matter removal, microbial community structure, bacteria, fungi.

INTRODUCTION

Forests are key ecosystems providing ecological and economic benefits (Constanza et al. 1997; Krieger, 2001). Ecological functions of forests are partially assumed by crucial role of these ecosystems on global carbon cycle (Lal, 2005; Pan et al., 2011). Indeed, forests contribute to mitigate climate change, annually capturing around 30% of atmospheric carbon dioxide derived from fossil fuel emissions (Canadell and Raupach, 2008) and sequestering almost 50% of total terrestrial carbon (Lal, 2005; Pan et al., 2011; Bar-On et al., 2018). Economic contributions of forests are also substantial, notably with the harvesting of woody biomass for materials and energy production (Kirschbaum, 2003; Achat et al., 2015). Ensuring high productivity as well as managing the carbon stock of forest lands has become a major concern. This concern has developed because forest ecosystems are currently submitted to intensive forest practices (Achat et al., 2015). Growing interest to wood fuel additionally with the increase of the competition for woody biomass between material and energy uses contribute to change in forest practices toward the harvest of residues such as foliages, branches and tree tops which were previously conventionally left in the forest (Diaz-Yanez et al., 2013; Repo et al., 2014; Di Fulvio et al., 2016). This intensive forest management contributes to increase wood production in comparison to conventional stem-only harvest practice but could negatively impacted forest ecosystem sustainability (Haberl et al. 2010; Bentsen & Felby, 2012). Recent meta-analysis revealed negative impacts of intensive forestry on soil carbon stock, soil fertility and tree productivity (Achat et al., 2015). If intensive forestry impact on soil abiotic parameters is well-studied, the effect on biotic soil component such as microorganisms remains less explored. By the mediation of key processes such as nutrients cycling, the soil microbiota regulates soil productivity and modulates forest ecosystems in response to stress (Barrios, 2007; Allison and Martiny, 2008; Singh et al. 2010). Microorganisms are involved in decomposition of organic matter that enables cycling of carbon and nutrients containing in dead animals, plant and microbial biomass (van der Heijden et al., 2008). Nutrients are made available by microorganism contributing to the primary productivity of forest ecosystems (Kyaschenko et al., 2017 b). However, our understanding of how microbial soil communities respond to intensive forest practices is poor. Large scale investigation of the impact of intensive forestry on microbial community using recent microbial community characterisation approach such as massive amplicons sequencing was mainly conducted on the Long-Term Soil Productivity (LTSP) experimental network. LTSP study regroups north American experimental sites with plots submitted to different degrees of OM removal following a clear-cut and compared to non-

harvested forest reference plots (Powers et al., 2005). Bacterial and fungal community structures drastically changed in the OM removal treatments in comparison to the non-harvested treatment (Hartmann et al., 2012; Wilhelm et al., 2017). Nevertheless, these equivocal results are strongly limited due to the fact the in LTSP network OM removal treatments comprise young forests while non-harvested forests are old forests. Indeed, forest age is well known to strongly drive microbial community composition (Pennanen et al., 1999; Banning et al., 2011; Hagenbo et al., 2018). In the LTSP sites, whole tree harvesting in comparison to stem-only harvesting appeared to have minor impact on microbial community structures (Hartmann et al., 2012; Wilhelm et al., 2017). Microbial community structure between stem-only harvesting and whole-tree harvesting treatments were significant in 5% of LTSP studied sites for bacterial communities and 12% of LTSP studied sites for fungal communities (Wilhelm et al., 2017). Poor OM removal effect on microbial communities were also noticed in studies conducted independently of the LTSP network (Busse et al., 2006; Hannam et al., 2006). These inconsistent results about intensive forest practices on fungal and bacterial community structures could indicate a strong resilience of soil microorganisms to intensive forestry. Nevertheless, effect of intensive forestry on microbial community composition could be delay and measurable after more than one intensive forestry tree rotation as it is generally studied. As forest rotation are generally long, some authors proposed to artificially remove forest OM to mimic long term impact of intensive forest practices (Melany et al., 2001). We investigated OM removal impact on forest soil communities at large scale on six organic matter manipulation sites across French temperate forest using bacterial (16 S rRNA gene) and fungal (ITS) phylogenetic gene marker libraries. We hypothesize (1) that OM removal would differentiate soil bacterial and fungal communities in harvested plots. We also hypothesize (2) that OM removal impact on bacterial and fungal community will be more important in the topsoil layer than in the subsoil layer. We finally hypothesize (3) that OM removal would increase relative abundance of stress tolerant and/or poor OM decomposers microbes.

MATERIALS AND METHODS

Study site, experimental design and soil sampling

The study was conducted in six OM manipulation sites belonging to the “Matières Organiques des Sols réseau” (MOS network) in France (Table 1). All the sites are dominated by *Fagus sylvatica*. Each site contained 12 plots (40 m × 40 m), which corresponded to four OM manipulation treatments repeated three times as described by Akroume et al. (2016). In this

study, we focused on two treatments: conventional French forest management (reference plot: REF) and yearly residue and litter removal (OM removal: OMR). The OMR treatment consisted of manually removing every late autumn all coarse woody debris and leaf litter using a power-driven 104 sidewalk sweeper and big packs. When we collected the soil samples, the OM removal treatment had been applied for three years. To limit the spatial heterogeneity (Akroume et al. 2016) at each of the six forest sites, we sampled on both sides of the boundary separating adjacent REF and OMR plots as described in Maillard et al. (submitted). As there is more than one side-by-side REF and OMR plot per site, we selected the pairs with the less soil heterogeneity based on the soil data published by Akroume et al. (2016). In September 2016, we sampled at each site nine soil cores (15 cm diameter) for each of the two treatments (n=108 soils cores) as shown in Figure 1. The separation into the topsoil (0-5 cm) and lower layer (5-10 cm) was performed in the field for each biological replicate (n=216 soils sampled). In the laboratory, the soil samples were sieved (2 mm) and froze. An aliquot of each soil sample was freeze-dried for DNA extraction.

Fungal and bacterial identification

We extracted total nucleic acids from 0.5 g of sieved soil (n=216 soil samples) using the FastDNA SPIN Kit for soil (MP Biomedicals, Solon, OH, USA) with the addition of 20 mg of Polyvinyl-polypyrrolidone (Sigma-Aldrich, France). We amplified fungal ITS1 region, recognized as DNA barcode for fungal identification, with the forward ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA) and reverse ITS2 (5'-GCTGCGTTCTTCATCGATGC) primers. We carried out amplification on 1 µL of template DNA in a volume of 25 µL using Taq PCR Master Mix Kit (QIAGEN). The program was as follow: 3 min at 94°C, 35 cycles of 1 min at 95°C, 1 min 52°C and 1 min at 72°C, and a final step 10 min at 72°C. We done The PCR reactions for each sample in triplicate and pcr products were confirmed using gel electrophoresis and pooled. To identify bacterial community, we amplified the V4 region of the 16S SSU rRNA with the forward 515F (5'-GTGCCAGCMGCCGCGGTAA) and reverse 806R (5'-GGACTACHVHHHTWTCTAAT) primers. We carried out amplification on 1 µL of template DNA in a volume of 25 µL using Taq PCR Master Mix Kit (QIAGEN). The program was as follow: 3 min at 94°C, 35 cycles of 1 min at 95°C, 1 min 50°C and 1 min at 72°C, and a final step 10 min at 72°C. We done the PCR reactions for each sample in triplicate and PCR products were confirmed using gel electrophoresis and pooled. Fungal and bacterial library were sequenced using MiSeq 2 × 250 bp V2 Illumina chemistry by the GeT-PlaGe platform (Auzeville, France). For bacterial and fungal communities, sequences were demultiplexed

according to their tags, filtered and trimmed (Quality score 30). Shorter sequences (<200 bp), as well as chimeric sequences and singletons that were removed based on prediction by Uchime, USEARCH v8.0.1616 software (Edgar, 2013). Dereplication and clustering were performed with USEARCH software. Operational taxonomic units (OTUs) were generated at 97% similarity threshold. Taxonomic assignment of representative sequences for each fungal OTU was done by using the Basic Local Alignment Search Tool (BLAST) algorithm v2.2.23 (Altschul et al., 1990) against the UNITE database (Kõljalg et al., 2013). For bacteria, sequences representative of each OTU were assigned to bacterial taxa using SILVA (Pruesse et al., 2007) at a confidence threshold of 97%. We obtained the total number of sequences corresponding to each fungal and bacterial OTUs. Fungal and bacterial OTUs number were rarefied to respectively 7690 and 4760 sequences per sample. Fungal OTUs were assigned to trophic status using FUNGuild (Nguyen et al., 2016). As main part of soil fungal OTUs lack clear trophic characterization with the exception of ectomycorrhizal fungi we defined three groups based on their growth form and their trophic status: “Filamentous ectomycorrhizal”, “Filamentous other” and “Yeast”. Finally, when possible, we manually assigned growth form and trophic status of OTUs that were unassigned mainly due to missing genus taxonomy or recent taxonomical name change.

Statistical analysis

Statistical analysis and data representation were performed on R software (R Core Team 2016) on bacterial and fungal rarefied counts. Species richness, Simpson index (H) and Pielou’s evenness (J) were calculated with Vegan package (Oksanen et al., 2013). We evaluated site, soil depth and OM removal treatment potential effect on species richness, Simpson index (H) and Pielou’s evenness (J) using three-way analyses of variance (ANOVA). For each site, species richness, Simpson index (H), Pielou’s evenness (J) were compared using two-way ANOVA with Tukey’s HSD test. Differences in soil fungal and bacterial OTUs composition were visualized with non-metric multi-dimensional scaling (NMDS) plots based on Bray-Curtis dissimilarity matrix using the metaMDS function in Vegan package. Permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis dissimilarity were applied on fungal and bacterial OTUs composition to determine site, soil depth and organic matter removal treatment potential effect. We also used PERMANOVA for fungal and bacterial OTUs composition analysis within each studied site. To determine fungal genera and bacterial classes responsible for the measured multivariate patterns we used Similarity Percentage analysis (SIMPER).

SIMPER analysis was associated with statistical analysis of treatment effect on fungal genera and bacterial classes using a mixed linear model with site as random factor with lme4 package (Bates et al., 2015). Treatment effect on fungal and bacterial community composition were represented with Heat trees and Heat maps using MetacodeR package (Foster et al., 2017). All statistical tests performed were considered significant using a threshold of $P < 0.05$.

RESULTS

Miseq sequencing

After bioinformatic step, seven bacterial samples presenting low number of sequences were removed before rarefaction step. Number of samples we retained per site per treatment and per depth are summarized in the supplementary Table 1. Finally, we obtain 1661040 ITS1 sequences for 216 samples representing 3233 fungal OTUs and 952000 16S sequences for 200 samples representing 2999 bacterial OTUs.

Impact of OM removal on soil bacterial communities

OM removal had minor effect on soil bacterial OTUs alpha diversity (Table 2). Analysis of variance of OTUs richness, H and J indices revealed no significant OM removal alteration of bacterial alpha diversity. We detected a significant Site*Treatment interaction, indicating that OM removal impact on bacterial alpha diversity was inconsistent between sites. Site by site alpha diversity analysis (Figure S1) exhibited a significant increase of bacterial species richness in the 0-5 cm of Verrière (VE) site in response to OM removal. PERMANOVA showed that OM removal had significant impact on bacterial OTUs composition explaining 1.5% of variation in distance for the six studied sites (Table 3). Due to huge site effect on bacterial community composition explaining 31% of variation in distance we analysed OM removal effect within each site. NMDS plots indicated a strong differentiation of bacterial communities in function of depth and treatment (Figure 1 a). NMDS analysis were confirmed by PERMANOVA which revealed that OMR treatment significantly changed bacterial OTUs composition at six studied sites in the 0-5 cm and at five studied sites in the 5-10 cm (Figure 1 b). Bray-Curtis dissimilarity indices calculation of bacterial OTUs composition indicated the OM removal effect were more important in the topsoil layer than in the subsoil layer. Compiègne (CO), Verrière (VE) and Gailfontaine (GA) sites presented the more important changes in bacterial OTUs composition in response to OM removal with Bray-Curtis dissimilarity. In terms of composition, soil bacterial communities were dominated by Acidobacteria, Proteobacteria, Actinobacteria and Verrucomicrobia, these four classes

representing almost 90% relative abundance for the six sites and for the two soil layers studied (Figure 1 c). Heat tree showed that the overall bacterial community structure was affected by OM removal in both 0-5 and 5-10 cm layers with the increase and decrease of bacterial taxa relative abundance by comparison to the REF treatment (Figure 2 a). In the 0-5 cm, relative abundance of Acidobacteria order significantly increased in response to OM removal contributing to 20% of total dissimilarity of bacterial community (Figure 2 b). Less abundant classes such as AD3, Acidimicrobiia, Actinobacteria, Thermoleophilia and Planctomycetacia relative abundance significantly increased in the OMR plots. By opposition, relative abundance of Alphaproteobacteria, Bacteroidia, Gammaproteobacteria, Verrucomicrobiae, Thermoanaerobaculia and Gemmatimonadates significantly decreased in the OMR plots by comparison to REF plots. In the 5-10 cm, relative abundance of AD3, Actinobacteria, Planctomycetacia and Deltaproteobacteria classes significantly increased in OMR plots. Alphaproteobacteria and Verrucomicrobiae relative abundance significantly decreased in response to OM removal. Heat tree representation displayed that OMR treatment impact on bacteria composition was qualitatively similar between 0-5 and 5-10 cm. Nevertheless, PERMANOVA and Bray-curtis indices showed that OM removal treatment on bacterial community structure was more important in the 0-5 cm than in the 5-10 cm soil layers. We used heat map representation based on OMR/REF fold change to identify abundant bacterial genus responding in the same way for the six studied sites for OM removal that we can consider as indicator of OM removal (Figure 3). For both 0-5 and 5-10 cm layers main part of abundant bacterial genera presented increase or decrease relative abundance in response to OM removal but only a few bacterial genera presented the same response for OM removal in all the studied sites. We noticed that *Aquisphaera* and *Mycobacterium* relative abundance increased in the OMR plots of the six studied sites at the 0-5 cm. *Reyranella* relative abundance decreased in the OMR plots of the six studied sites in the 0-5 cm. In the 5-10 cm, we observed an increase of *Granulicella* and *Burkholderia* relative abundance in all the OM removal plots.

Impact of OM removal on soil fungal communities

OM removal influenced significantly fungal OTUs richness ($P=0.04191$), H ($P= 0.0196$), J ($P= 0.03745$) indices (Table 2). We measured an overall decrease of fungal alpha diversity in response to OM removal but site by site analysis revealed poor significant effect of OM removal (Figure S2). PERMANOVA analysis we conducted on overall fungal data revealed that OM removal significantly impacted fungal OTUs composition explaining 1.3% variation in distance (Table 3). NMDS plots for each of the six studied sites showed a clear clustering of fungal

OTUs composition in function of depth (Figure 4 a). OM removal treatment on fungal OTUs composition visualized with NMDS appeared to be site dependant especially in the subsoil layer (5-10 cm) which was confirmed by pair-wise PERMANOVA analysis (Figure 4 b). OTUs fungal community composition significantly differed between OMR and REF plots in the 0-5 cm for five of the six studied sites. Bray-Curtis dissimilarity indices between OMR and REF treatment for the 0-5 cm ranged from 0.43 to 0.61. OM removal appeared to have less impact in the subsoil layer with only two studied sites presented a significant change of fungal community structure. Bray-Curtis dissimilarity indices between OMR and REF treatment for the 5-10 cm soil layer ranged from 0.41 to 0.66. Fungal communities were dominated by Basidiomycota phylum ranged from 40 to 70% relative abundance (Figure 4 c). Basidiomycota were mainly represented by Agaricomycetes class. Zygomycota represented 5-25% of relative abundance depending of site and were mainly composed of Mortierellomycetes and Umbelopsidomycetes classes. Finally, Ascomycetes phylum representing 1 to 15% of relative abundance mainly dominated by the Leotiomycetes class. SIMPER analysis showed that *Russula* genus increased in OM removal plots contributed to explain respectively 20 and 23% of dissimilarity between OMR and REF treatment for 0-5 and 5-10 cm. But, *Russula* relative abundance did not differed significantly in response to OM removal which was caused by high site response variability to OM removal (Figure S3). Heat tree analysis conducted on the overalls six sites OTUs compositions exhibited OM removal impact on fungal composition with increase and decrease of fungal taxa (Figure 5). For both 0-5 and 5-10 cm we measured a significant increase of *Laccaria*, *Inocybe* and *Saitozyma* and a decrease of *Mortierella* and *Xerocomellus* relative abundances. At the genus level, OM removal impact appeared to be very similar between top and subsoil layers which is also showed by heat trees representation except for Helotiales fungi. We observed that Helotiales fungi increased in response to OM removal in the topsoil layer and decrease in the subsoil layer. Functional assignment of fungal community revealed a significant increase of ectomycorrhizal and yeast fungi in response to OM removal and a decrease of saprotroph filamentous fungi and other and unclassified fungi. These results were mainly notable in the topsoil layer. We detected a limited number of abundant fungal OTUs considered as indicator of OM removal. Fold change based heat map analysis revealed that two OTUs, *Clavulina_sp* and *Mortierella_sp* decreased in OMR plots in the 0-5 cm of the six studied sites. *Russula romelii* relative abundance which was detected in three of the six studied sites increased in response to OM removal in both 0-5 and 5-10 cm layers.

Link between soil parameters and intensity of microbial community response to OM removal.

We applied Pearson's correlation to identify potential drivers of bacterial and fungal community response to OM removal. We measured no significant correlation between soil site parameters and bacterial and fungal OM removal impact on alpha and beta diversity (Figure S5).

DISCUSSION

Organic matter removal impact on soil bacteria

After three years of leaf litter and dead wood removal we revealed that no major large-scale effect of OM removal treatment on bacterial alpha diversity. These findings are in agreement with other OM manipulation studies revealing strong resilience of soil bacterial community diversity to disturbance (Busse et al., 2006; Hannam et al., 2006; Hartmann et al., 2008; 2012; Wilhelm et al., 2017). Nevertheless, three years of OM removal initiated a strong bacterial community restructuring in both top and subsoil layers respectively significant in 100% (6/6) and 80% (5/6) of studied sites confirming our hypothesis (1). However, our results are in contradiction with other findings showing that intensive forestry impact didn't affect soil bacterial community in comparison to stem only harvesting (Hartmann et al., 2008; 2012; Wilhelm et al., 2017). Nevertheless, other studies indicated a shift in soil bacterial communities in response to whole-tree harvesting (Mushinski et al., 2018) and in response to leaf litter removal (Leff et al., 2012). As we hypothesised (2), bacterial communities were less impacted by OM removal in subsoil layer which is consistent with previous study (Mushinski et al., 2018). We measured significant expansion of bacterial orders well defined by meta-analysis and large-scale correlation approaches as oligotrophic such as the Acidimicrobia, the Actinobacteria and the Acidobacteria in the OMR plots (Fierer et al., 2007; Tian et al., 2018). By opposition, we measured significant decline of bacteria orders defined as copiotrophic such as Alphaproteobacteria, Gammaproteobacteria and Verrucomicrobiae, Bacteroidia, Gemmatimonadates in the OMR plots. Leff et al. (2012) observed a strong increase of Acidobacteria abundance in response to leaf litter removal. Together, these results suggest that Acidobacteria, one of the most abundant bacterial classes in soil, is a robust indicator of OM manipulation disturbance. Soil copiotrophic bacteria are generally correlated with soil organic matter content (Fierer et al., 2007; Tian et al., 2018). By decreasing input of plant OM, we could have induced a decrease of copiotrophic bacteria in favour of oligotrophic bacteria which are

generally more able to deal with low soil OM content. These results supported our hypothesis (3) and strongly indicate a restructuration of bacterial in response to OM removal in favour of oligotrophic bacterial populations. In a previous study we revealed no decrease of soil OM in response to OM removal in our studied sites (Maillard et al., submitted). These results indicate that change in bacterial community structure in favour to oligotrophic bacterial populations is a more sensible indicator of OM removal than the direct measurement of soil OM content. We identified at low taxonomic level bacterial indicators of OM removal disturbance such as *Aquisphaera*, *Mycobacterium*, *Reyranella*, *Granulicella* and *Burkholderia*. Similarly, Mushinski et al. (2018) depicted an increase of *Burkholderia* bacterial genus in response to intensive forestry which is consistent with our observations. Increase of *Burkholderia* relative abundance in response to OM removal could be explained by the important metabolic versatility of this genus as proposed by Mushinski et al. (2018). Moreover, bacteria belonging to the *Burkholderia* genus were also described as efficient mineral weathering and phosphate solubilizing bacteria in forest soils (Calvaruso et al., 2013; Colin et al., 2016; Nicolitch et al., 2016). As soil phosphorus content strongly decrease in the studied sites in response to OM removal (Maillard et al., submitted), high ability of *Burkholderia* to solubilize phosphorus could give to this bacterial genus a nutritional advantage explaining their expansion in the OM plots subsoil.

Organic matter removal impact on soil fungi

OM removal effect on fungal alpha diversity was minor as it was already showed (Busse et al., 2006; Hannam et al., 2006; Hartmann et al., 2008; 2012; Wilhelm et al., 2017). Fungal community composition significantly changed in response to OM removal plots in the topsoil in 80% (5/6) of the studied sites which confirmed our hypothesis (1). In the subsoil layer, OM removal impact was less important with significant fungal community structure between REF and OMR plots noted in 33% of the studied site (2/6). As we proposed in hypothesis (2) fungal community changes were mainly notable in topsoil layer. We observed significant expansion of *Laccaria* and *Inocybe* relative abundance in response to OM removal. These two fungal genera are intensively described as forest early-stage ectomycorrhizal fungi (Newton et al., 1992; Visser et al., 1995; Kranabetter et al., 2005; Twieg et al., 2007). By opposition, *Piloderma*, *Tricholoma* and *Xerocomellus* ectomycorrhizal genera, generally defined as forest late-stage ectomycorrhizal fungi, tended to decrease in response OM removal. Together, these results highlighted that in response to intensive OM removal in mature forest, ectomycorrhizal fungal community restructured in favour to young forest associated fungi populations. We observed a strong decrease of *Mortierella* genus in the OM removal plots by comparison to

REF plots. These results could indicate that *Mortierella* are implicated in soil organic matter degradation. If the role of *Mortierella* are not well defined, strong increase of this genera measured in response to organic amendment support our proposition (Li et al., 2018). We observed a significant increase of ectomycorrhizal fungi and yeast fungi relative abundance in response OM removal. Ectomycorrhizal fungi increase in the OM removal plots could be due to a decrease of soil saprotrophic fungi in response for the decrease of their C substrates input. These results are supported by an increase of seedling infections by ectomycorrhizal fungi in response to leaf litter removal observed in many studies (Baar & De Vries, 1995, Baar & Kuyper, 1993, 1998; Baar & Ter Braak, 1996). In a competition context, we could imagine that ectomycorrhizal fungi, receiving their C from their associated host plant, become more competitive against soil saprotrophic fungi when OM input decrease which is in accordance with our hypothesis (3). Identification of fungal OTUs indicators of OM removal remains limited in our study to few fungal genera. *Mortierella_sp* and *Clavulina_sp* responded in the same way in the topsoil of the six sites but their fold change remained minimal. For instance, *Russula romelli* relative abundance strongly increased in response to OM removal in both top and subsoil layers but was only detected in three of the six studied sites. Tyler (1991) already reported an increase of *Russula* carpophore in response to leaf litter removal. Nevertheless, this ectomycorrhizal fungus could be a good indicator of OM removal.

Organic matter removal shape microbial community toward stress tolerant and less efficient OM degrader populations.

We measured a restructuration of bacterial community toward oligotrophic bacteria in response to OM removal which are describe as poor OM decomposers (Fierer et al., 2007; 2012). Additionally, ectomycorrhizal fungi and yeast fungi which expanded in response to OM removal are generally also described as poor plant OM degraders (Colpaert & van Laere 1996; Kohler et al. 2015; Mašínová et al., 2018). Together, these results indicate a shift of microbial communities in OM plots toward less efficient OM decomposer microbes. If we extended our result to intensive forestry practices, we could imagine a decrease of degradation rate of belowground plant OM such as fines roots. This assumption is supported by strong decrease of soil enzymes activities and substrates catabolic activities of microbial communities measured in the OMR plots of six studied sites by Maillard et al. (submitted). Maillard et al. (submitted) also depicted an increase of fine roots surfaces in subsoil layers in OM removal plots. Thus, we proposed that OM removal by eliciting a shift in soil microbial community toward less efficient decomposers and an increase of plant fine roots surface could stabilize the C storage

in soil layers of OM removal plots at mid or long term. If C stock in forest floor clearly decreased in response to intensive forestry, results of C stock in soil appeared to be often inconsistent (Achat et al., 2015 a). Our assumption could support these results. Mid and long-term monitoring of C stock and microbial communities of the MOS network sites are now necessary to confirm our proposition. One limitation for this assumption is due to the fact that ectomycorrhizal fungi could have OM degradation abilities (Rineau et al., 2012; Bödeker et al., 2014; Lindahl & Tunlid 2015; Kvaschenko et al., 2017 a; Op de Beeck et al., 2018). Consequently, we must clearly identify the potential role of temperate forest EcM fungi in OM degradation using pure culture functional characterisation approach or ectomycorrhizae and soil metatranscriptomic analyses because field experiment associated with correlation approaches remained inconsistent (Kvaschenko et al., 2017 b). If intensive OM harvesting could have no effect on soil C stock content based on our assumption and recent measurement of SOM contents in the OMR plots (Maillard et al., submitted), nutrients contents strongly decreased and notably the phosphorus as described by Maillard et al. (submitted). Decrease of nutrients content due to OM removal combined to a decrease of OM recycling by microorganisms may cause strong fertility issue.

CONCLUSION

Our large-scale molecular results indicate that soil bacterial and fungal communities manifest significant responses to OM removal in beech tree dominated temperate forests. If alpha diversity was a poor indicator of OM removal perturbation microbial structure was clearly affected by this disturbance. Microbial communities proved to be sensitive indicators of environmental change associated with OM removal. We measured a shift in the main bacterial classes with an expansion of soil oligotrophic bacteria in OM removal plots concomitantly with a decrease of copiotrophic bacteria. Fungal communities presented a strong restructuration toward symbiotic and stress tolerant fungi populations in response to OM removal. The next step will be to explore consequences of this measured shift in microbial communities on soil properties and tree productivity at mid and long term.

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Conflict of interest

We state that there is no conflict of interest.

Tables

TABLE 1. Organic matter manipulation experimental sites description

Forest	Code	Species	First manipulation	Sampling		Coordinate	GPS Lambert93		Elevation	Humus type	Soil texture	Soil types (WRB 2014)
				date	Years		dirN	dirE				
Verrière du Grosbois	VE	Beech	16/05/14	Sept. 2016	55	6683070.9	948588.4	585	Mesomull/oligomull	Silty clay loam	Dystric Luvic Cambisols	
Gaillefontaine	GA	Beech	17/10/14	Sept. 2016	40	6952055.8	602895.7	290	Eumull/mesomull	Silty-loam	Eutric Cambisols	
Darney	DA	Beech	25/01/14	Sept. 2016	35	6779652.2	936520.6	500	Mesomull/oligomull	Sandy clay loam	Dystric Cambisols	
Compiègne	CO	Beech	25/04/14	Sept. 2016	30	6914427.8	695219.1	185	Mesomull	Sandy-loam	Stagnic Luvisols	
Ban d'Harol	BA	Beech	20/06/14	Sept. 2016	35	6783156.6	938890.7	350	Mesomull	Loam	Stagnic Dystric Cambisols	
Saint-Quirin	SQ	Beech	27/09/14	Sept. 2016	35	6842790.239	996528.371	400	Mesomull/dysmull	Sandy-loam	Dystric Cambisols	

TABLE 2. Three-way ANOVA with the influence of site, depth and treatment and their interactions, in the bacterial and fungal alpha diversity.

	Bacteria						Fungi					
	Species richness		H		J		Species richness		H		J	
	F value	p value	F value	p value	F value	p value	F value	p value	F value	p value	F value	p value
Site	5.826	0.001***	8.718	0.001***	11.076	0.001***	2.919	0.01452*	7.031	0.001***	8.778	0.001***
Treatment	0.684	0.4094	1.144	0.2862	1.474	0.22628	4.195	0.04191*	5.535	0.0196*	4.391	0.03745*
Depth	73.619	0.001***	69.13	0.001***	42.510	0.001***	161.070	0.001***	31.900	0.001***	11.670	0.001***
Site*Treatment	2.656	0.024*	3.025	0.0119*	3.541	0.00441**	2.665	0.02356*	1.437	0.2127	1.138	0.341798
Site*Depth	3.087	0.0106*	2.67	0.0234*	1.765	0.12196	3.638	0.00362**	1.310	0.2612	0.807	0.546074
Treatment*Depth	0.914	0.3404	1.463	0.228	0.905	0.3426	0.277	0.59938	0.222	0.6384	0.443	0.506561
Site*Treatment*Depth	1.816	0.1116	1.208	0.3071	0.377	0.8644	1.368	0.23797	0.418	0.8357	0.377	0.863799

Values in the table represent the F-ratio and the level of significance (*P≤0.05; **P≤0.01; ***P≤0.001).

TABLE 3. PERMANOVA with the influence of site, depth and treatment and their interactions, in the bacterial and fungal OTUs compositions based on Bray-Curtis dissimilarity matrix.

	Bacteria		Fungi	
	R2	P	R2	P
Site	0.32611	0.001***	0.18211	0.001***
Treatment	0.01528	0.001***	0.01368	0.001***
Depth	0.07325	0.001***	0.01624	0.001***
Site*Treatment	0.03775	0.001***	0.05300	0.001***
Site*Depth	0.04582	0.001***	0.02161	0.061
Treatment*Depth	0.00479	0.035*	0.00319	0.645
Site*Treatment*Depth	0.01301	0.463	0.00772	1.000

Values in the table represent the proportion of distances explained (R2) and the level of significance (*P≤0.05; **P≤0.01; ***P≤0.001).

FIGURE CAPTIONS

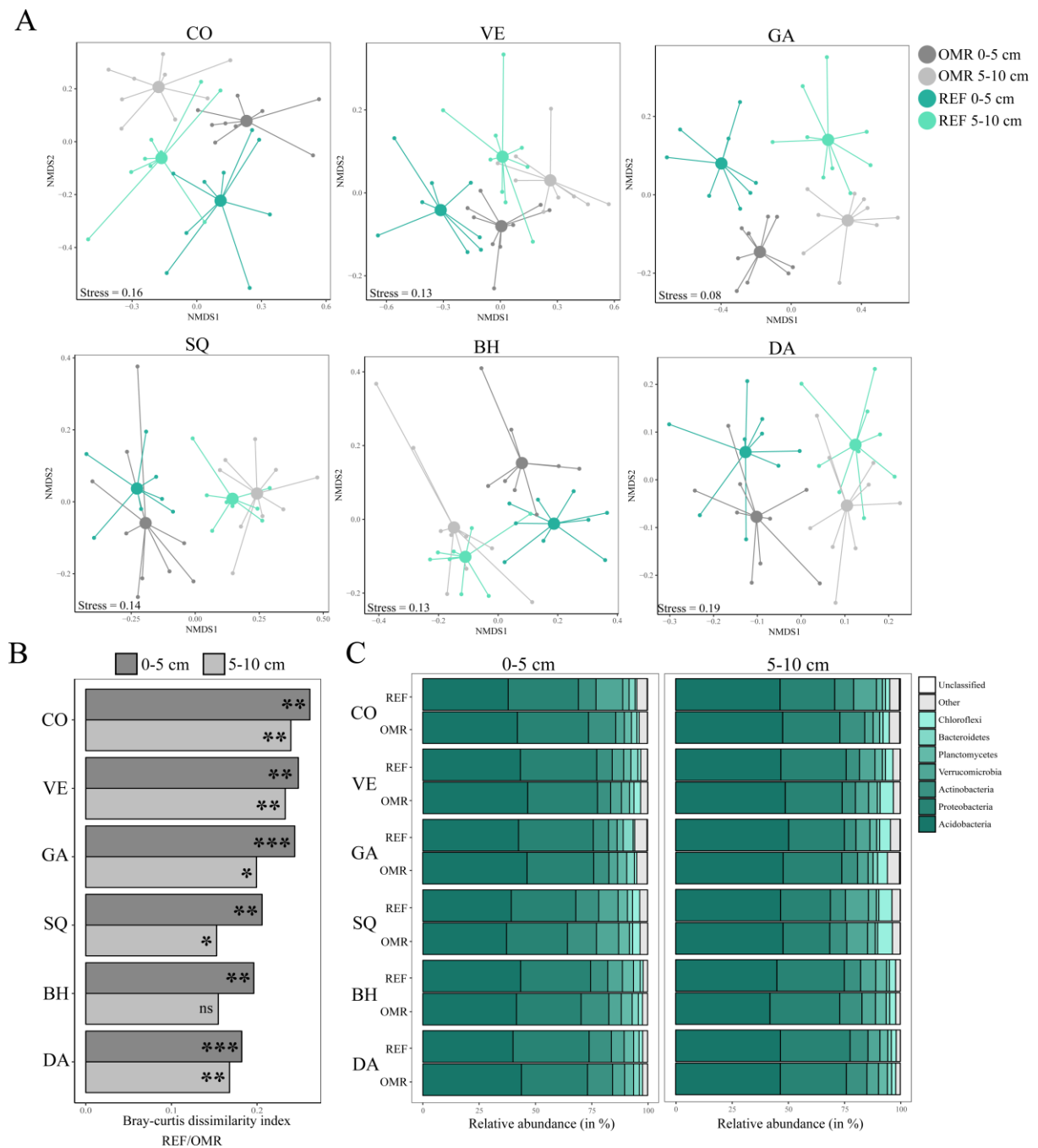


FIGURE 1. a Non-metric multidimensional scaling (NMDS) analysis of soil bacterial community based on bacterial OTUs abundance and Bray-Curtis dissimilarity matrix for each of the six studied sites coloured in function of treatment (REF and OMR) and soil depth (0-5 and 5-10 cm). b Bray-Curtis dissimilarity index between REF and OMR treatment were calculated with bacterial OTUs abundance for each of the six studied sites in function of soil depth (0-5 and 5-10 cm). Significant differences between bacterial community at the OTUs taxonomic level between REF and OMR treatment for each of the six studied sites in function of soil depth (0-5 and 5-10 cm) were calculated with PERMANOVA based on Bray-Curtis dissimilarity matrix and stars indicate the level of significance (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$). c Mean bacterial community composition at the class taxonomic level in function of site, depth and treatment.

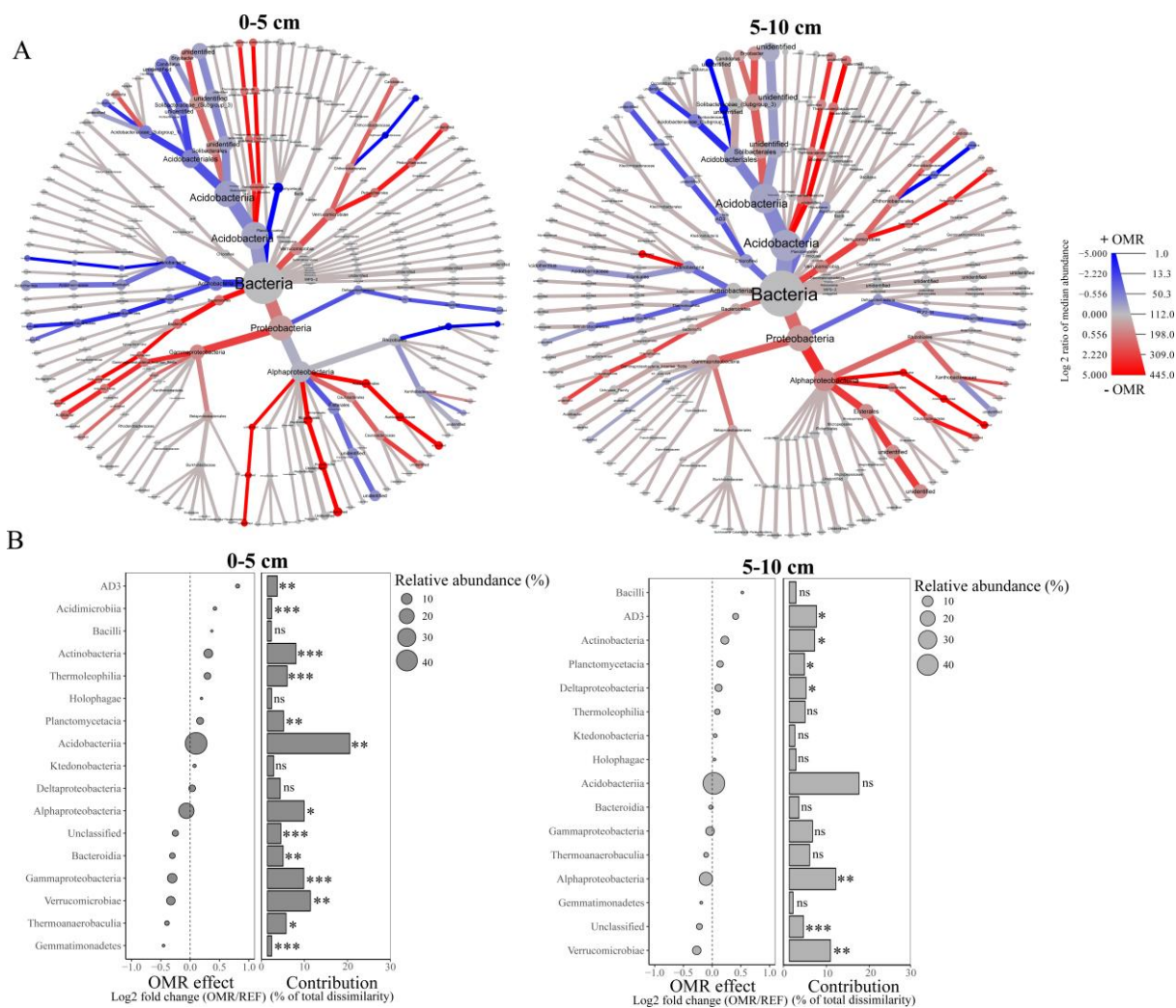


FIGURE 2. (a) Overall differential analysis of bacterial community composition of OM removal impact in function of soil depth (0-5 and 5-10 cm). Colour indicates the sign and intensity of averaged changes in relative bacterial abundance and the size indicates the bacterial relative abundance. (b) SIMPER analysis identifying the % contribution of bacterial classes representing at least 1% of contribution to the Bray-Curtis dissimilarity matrix between REF and OMR treatments in function of soil depth (0-5 and 5-10 cm). Significant effect of treatment on bacterial classes was evaluated with mixed linear model with site as random factor, stars indicate the level of significance (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

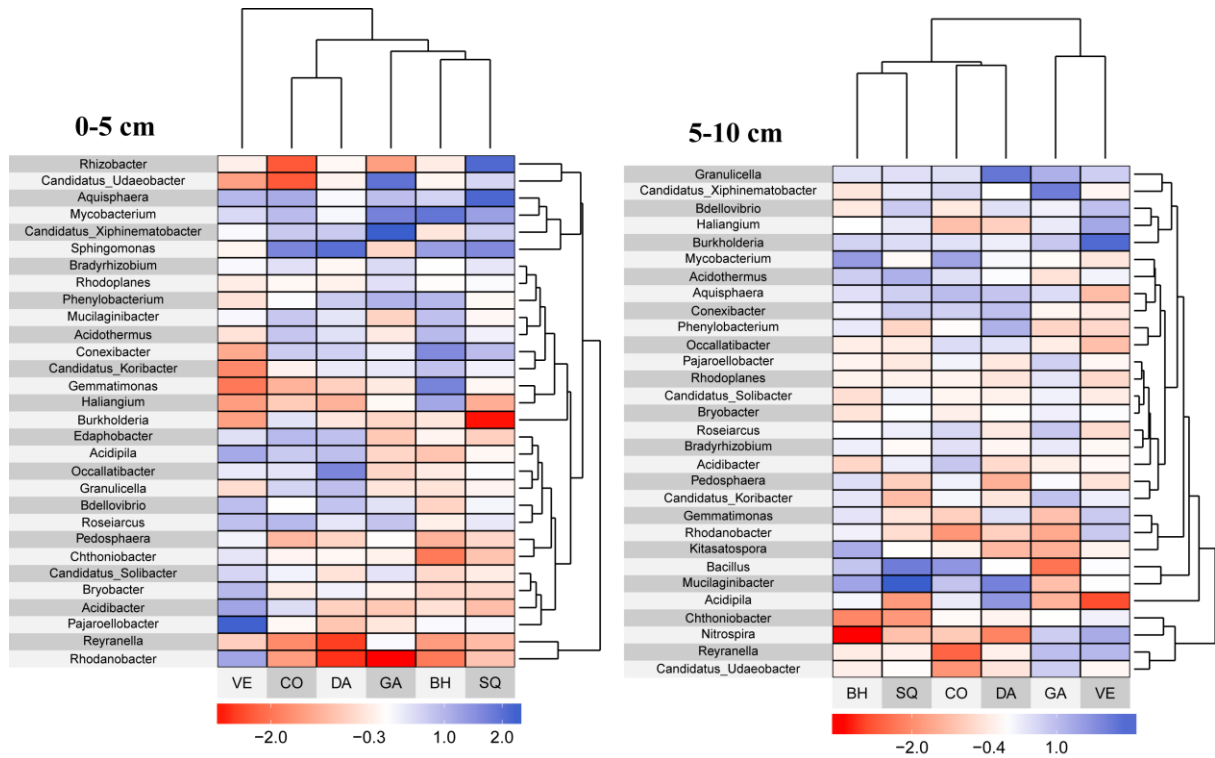


FIGURE 3. Impact of OM removal (log₂ fold change OMR/REF) on the 30 most abundant bacterial genera in function of site and depth (0-5 and 5-10 cm).

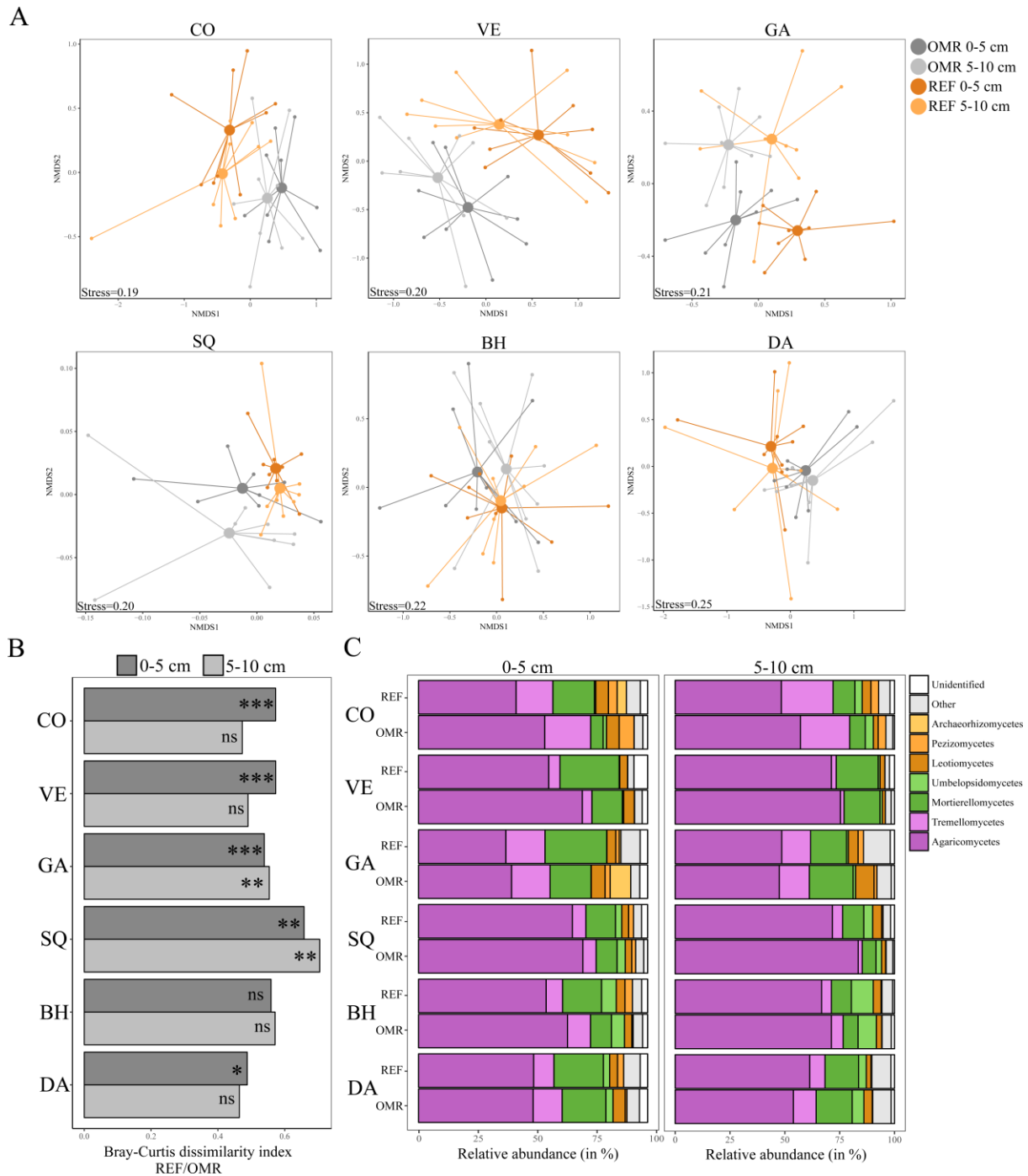


FIGURE 4. (a) Non-metric multidimensional scaling (NMDS) analysis of soil fungal community based on fungal OTUs abundance and Bray-Curtis dissimilarity matrix for each of the six studied sites coloured in function of treatment (REF and OMR) and soil depth (0-5 and 5-10 cm). (b) Bray-Curtis dissimilarity index between REF and OMR treatment calculated with fungal OTUs abundance for each of the six studied in function of soil depth (0-5 and 5-10 cm). Significant differences between fungal community at the OTUs taxonomic level between REF and OMR treatment for each of the six studied in function of soil depth (0-5 and 5-10 cm) were calculated with PERMANOVA based on Bray-Curtis dissimilarity matrix and stars indicate the level of significance (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$). (c) Mean fungal community composition at the class taxonomic level in function of each site, depth and treatment.

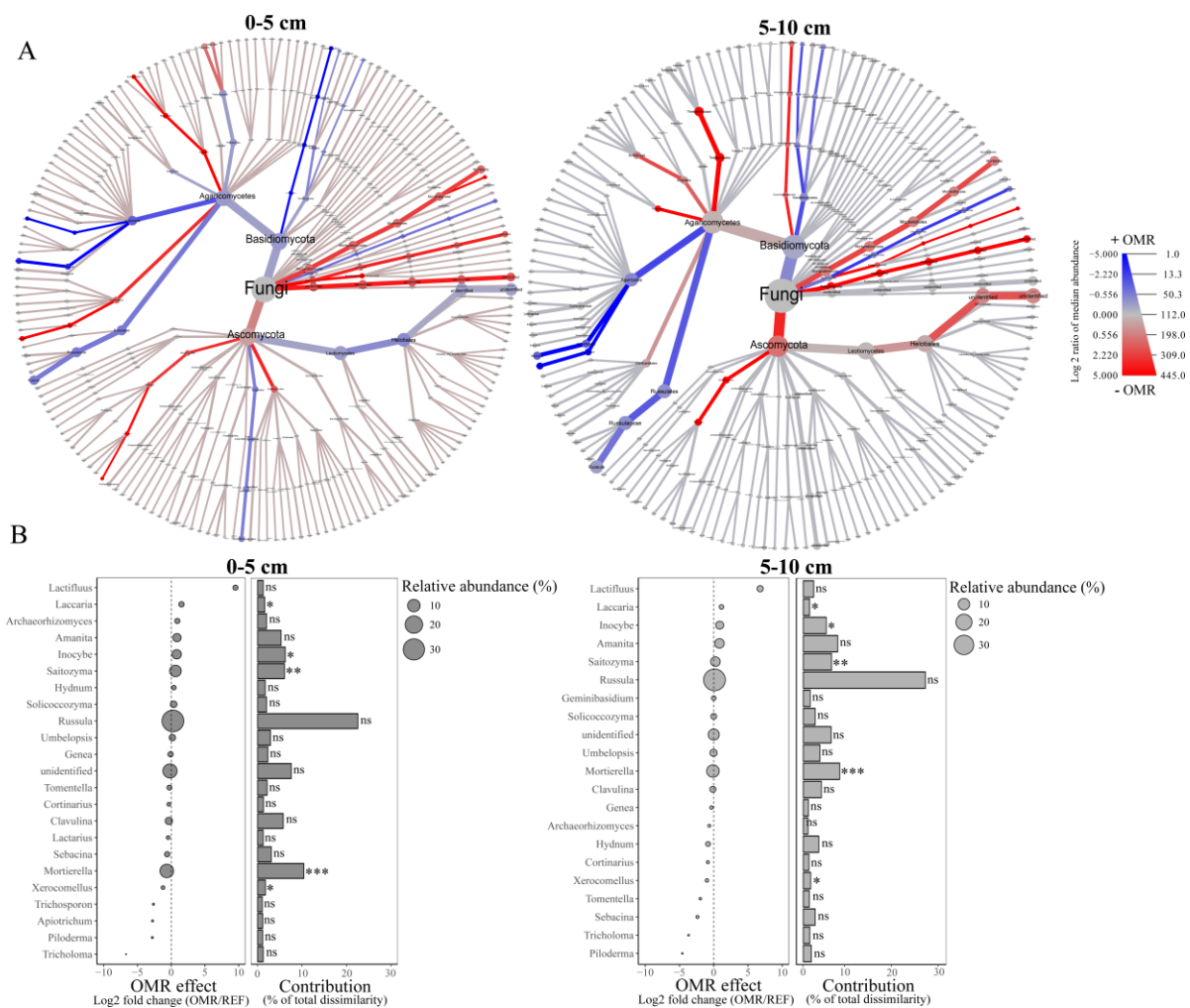
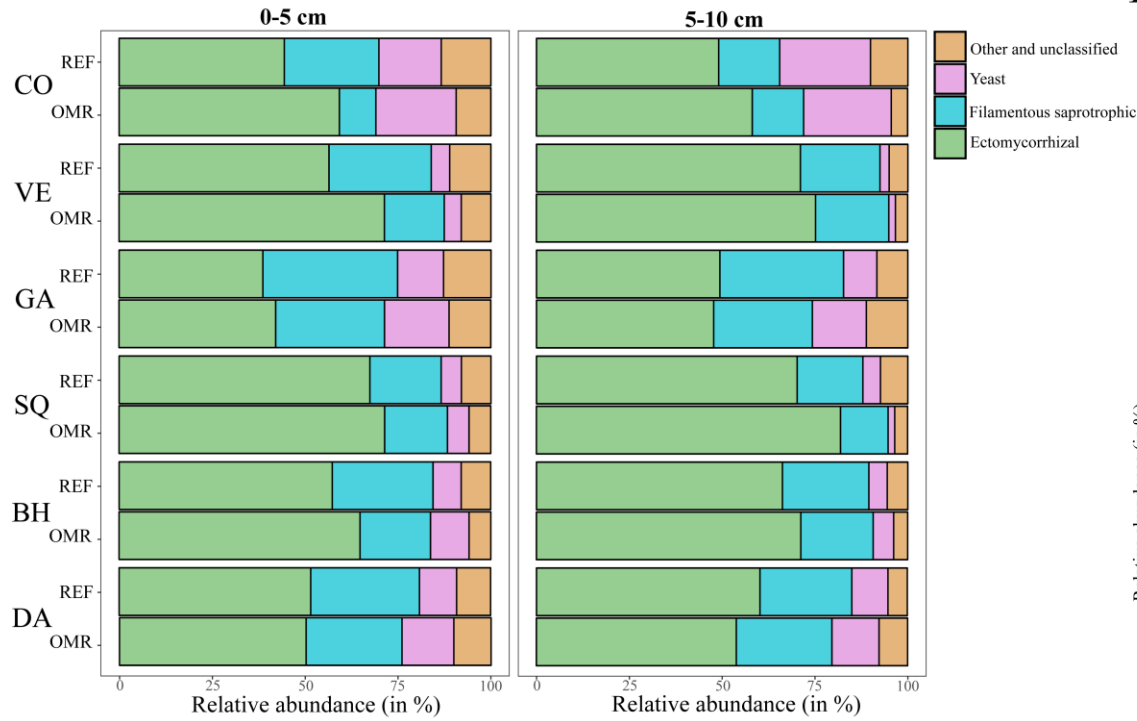


FIGURE 5. (a) Overall differential analysis of fungal community composition of OM removal impact in function of soil depth (0-5 and 5-10 cm). Colour indicates the sign and intensity of averaged changes in relative fungal abundance and the size indicates the fungal relative abundance. (b) SIMPER analysis identifying the % contribution of fungal genera representing at least 1% of contribution to the Bray-Curtis dissimilarity matrix between REF and OMR treatments in function of soil depth (0-5 and 5-10 cm). Significant effect of treatment on fungal genera relative abundance was evaluated with mixed linear model with site as random factor, stars indicate the level of significance (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

A



B

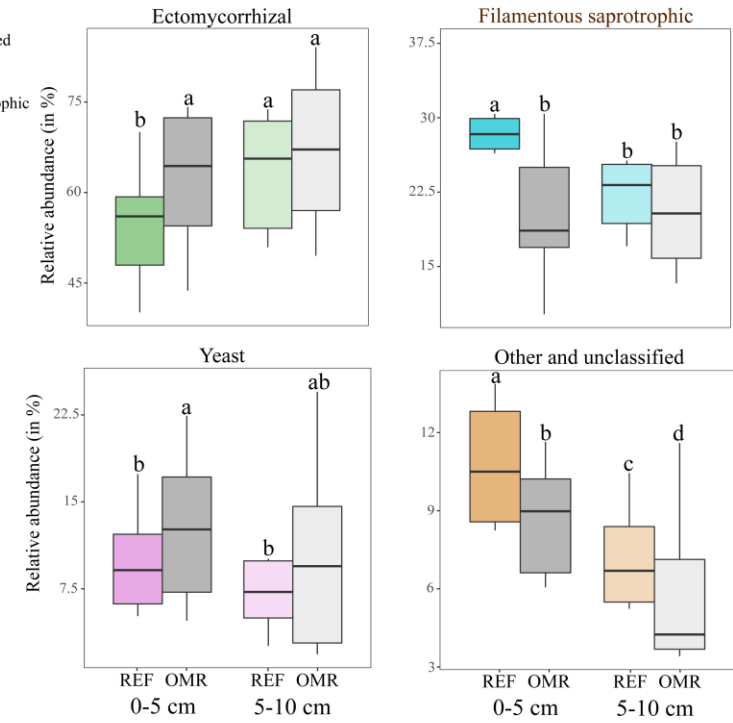


FIGURE 6. (a) Relative abundance of fungal guilds in function of site, depth and treatment. (b) Overall impact of OM removal on relative abundance of fungal guilds was evaluated using mixed linear model with site as random factor, different letters indicate significant differences.

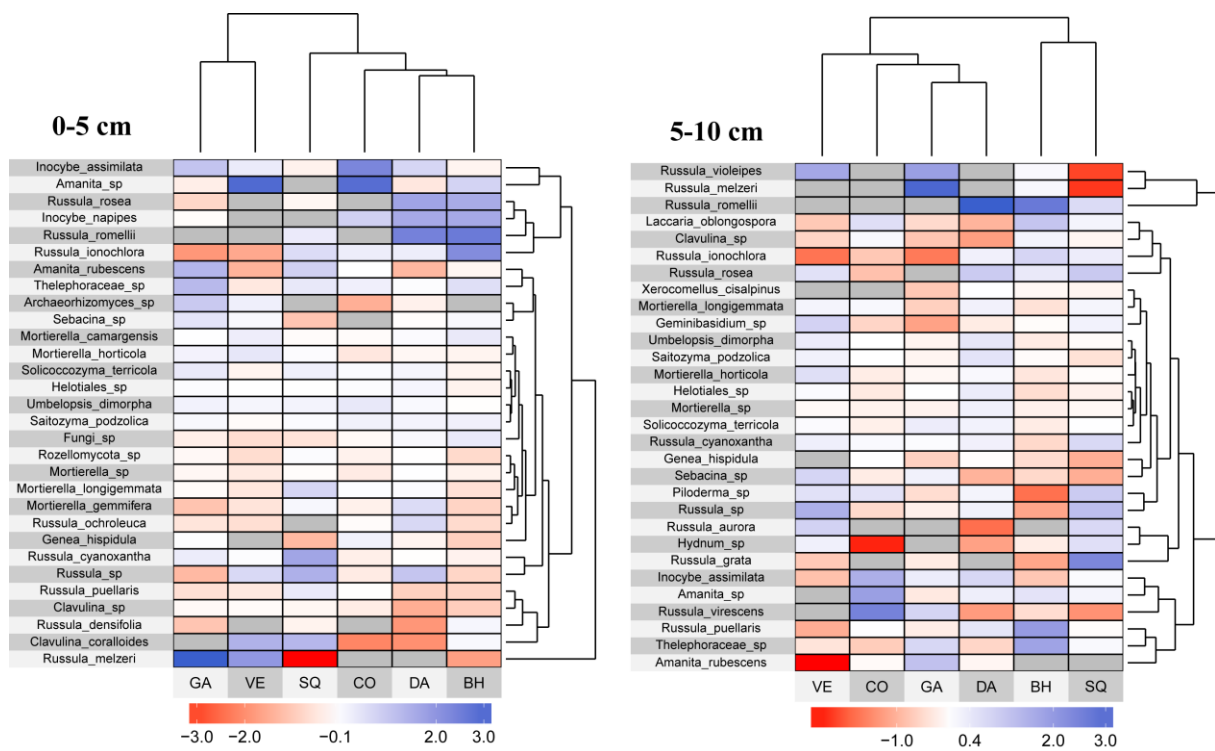


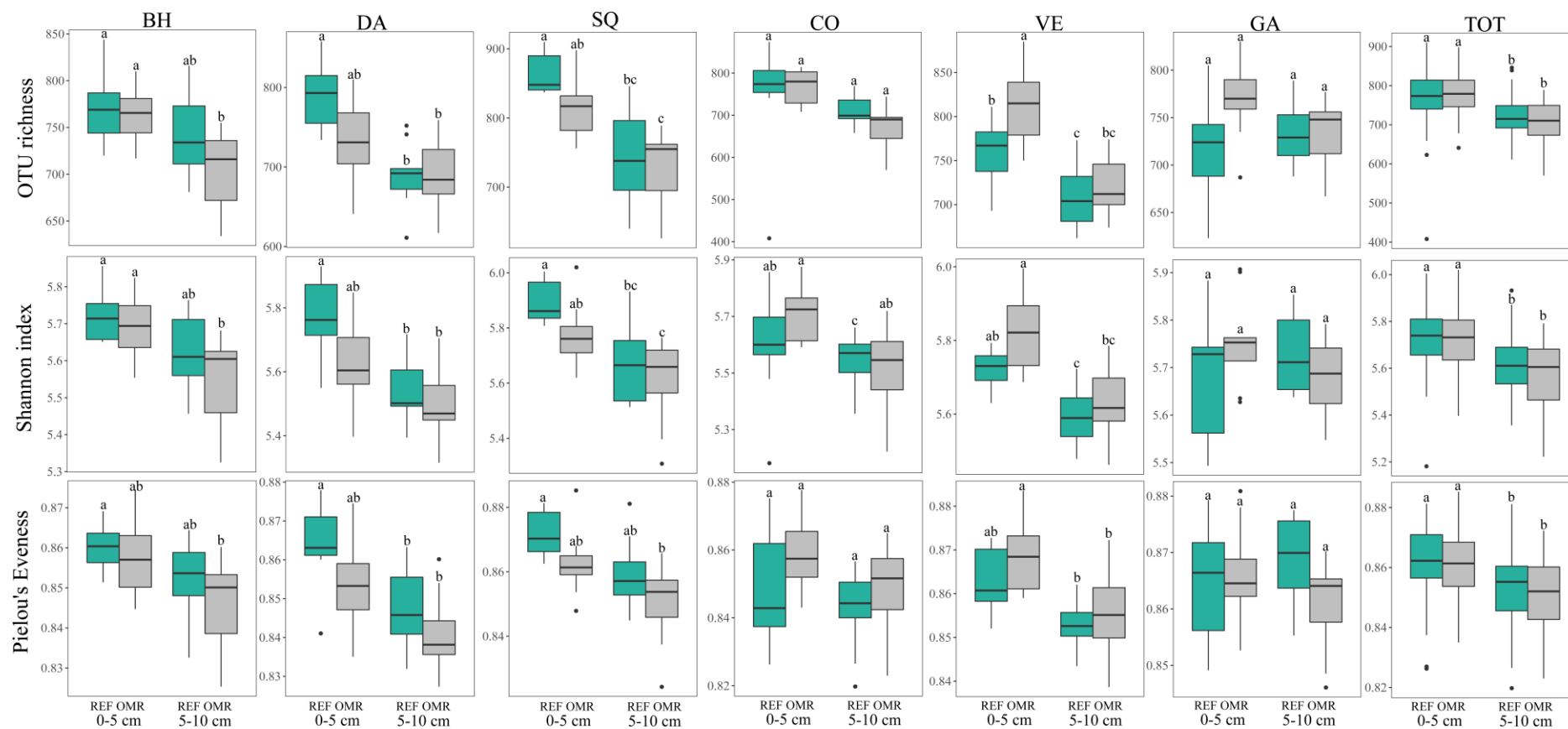
FIGURE 7. Impact of OM removal (log₂ fold change OMR/REF) on the 30 most abundant fungal OTUs in function of site and depth (0-5 and 5-10 cm).

Supplementary table

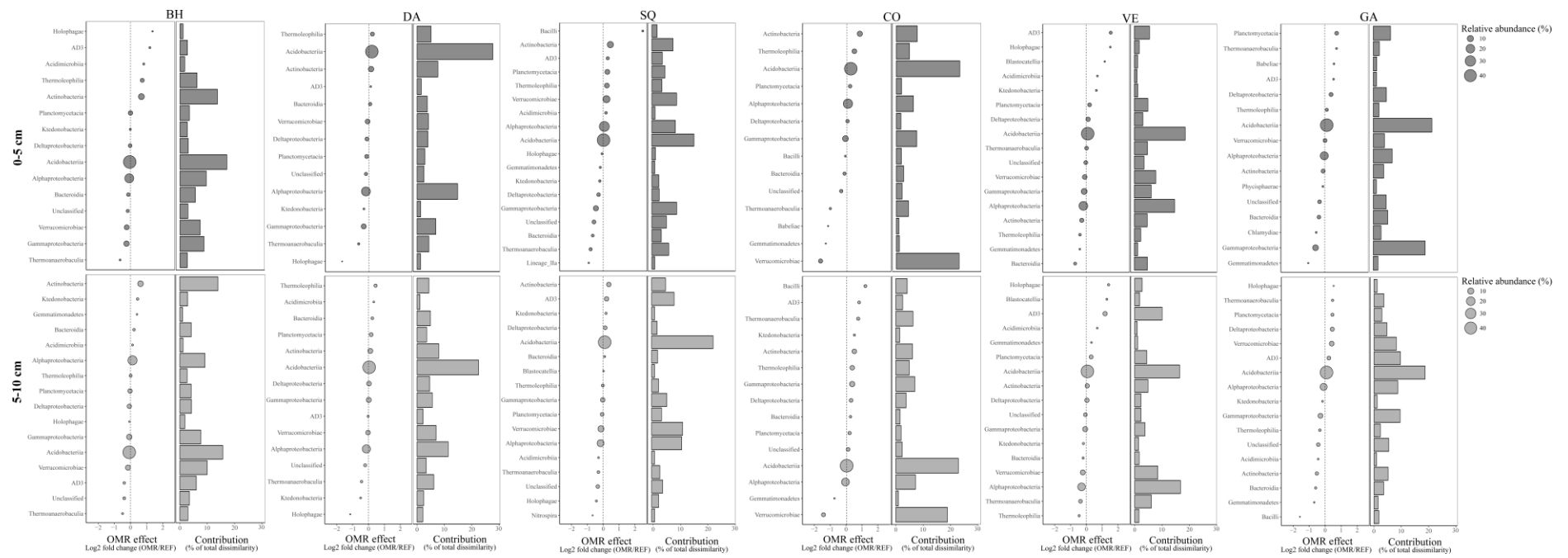
SUPPLEMENTARY TABLE 1. Number of biological replicates for bacterial and fungal community analysis in function of site, depth and treatment.

	0-5 cm				5-10 cm			
	REF		OMR		REF		OMR	
	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria
VE	9	8	9	9	9	9	9	9
GA	9	8	9	9	9	9	9	9
DA	9	9	9	9	9	9	9	9
CO	9	9	9	9	9	9	9	9
BH	9	9	9	8	9	9	9	9
SQ	9	7	9	9	9	8	9	9

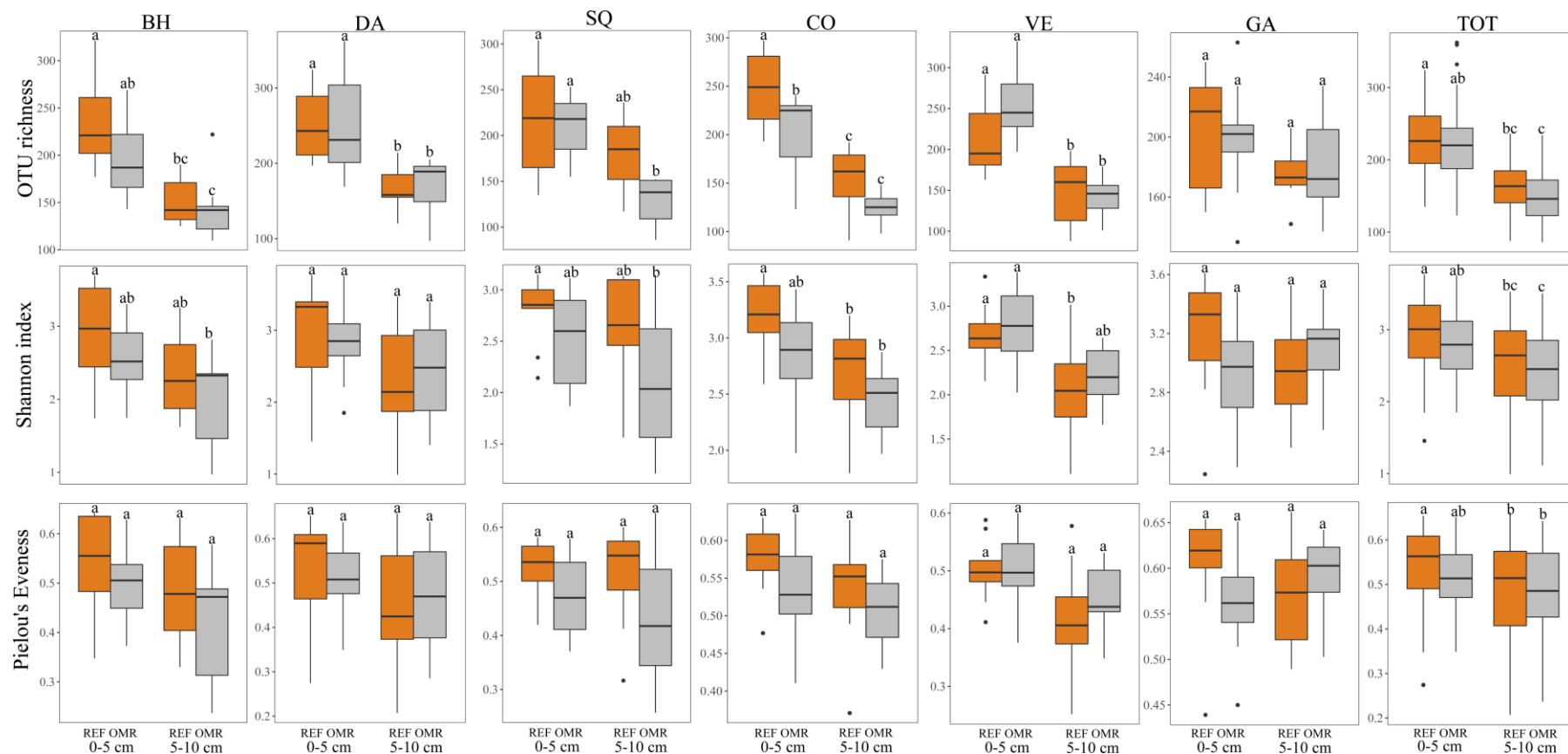
Supplementary data



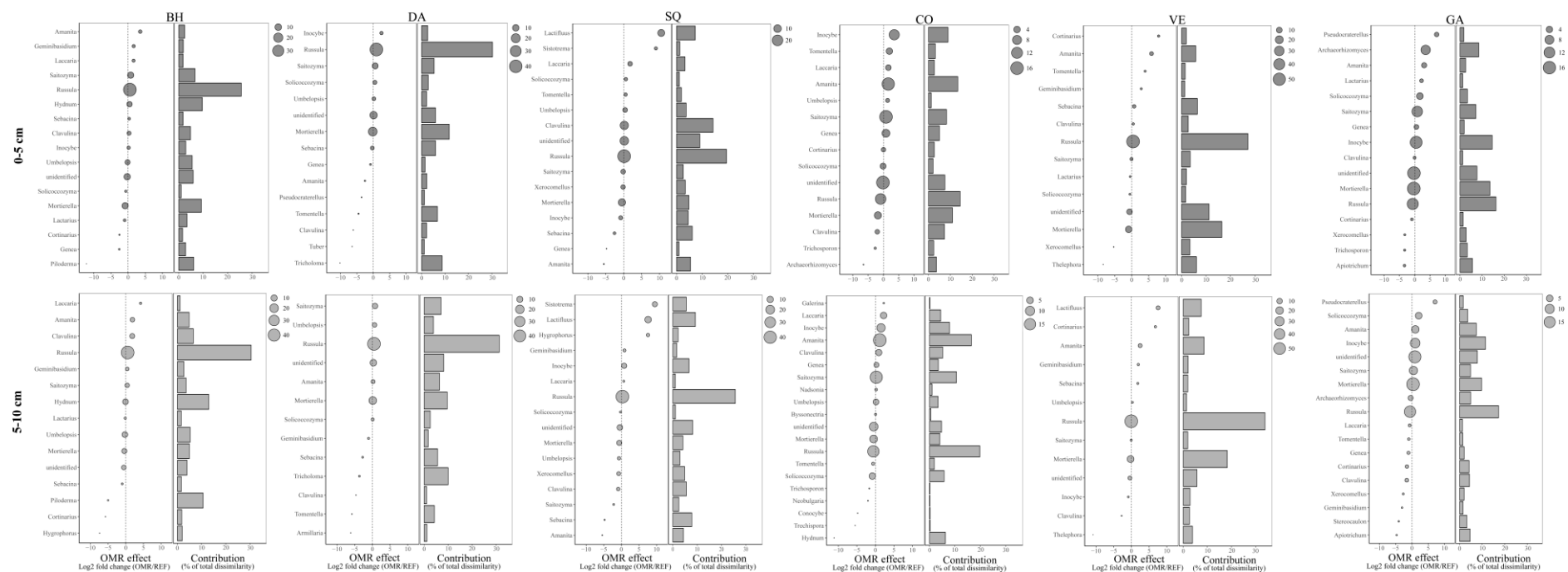
SUPPLEMENTARY FIGURE 1. Impact of OM removal on bacterial alpha diversity for each of the six studied sites. OM impact on bacterial species richness, Shannon index and Pielou's evenness were compared using two-way ANOVA and Tukey's HSD post hoc tests. Different letters indicate significant differences.



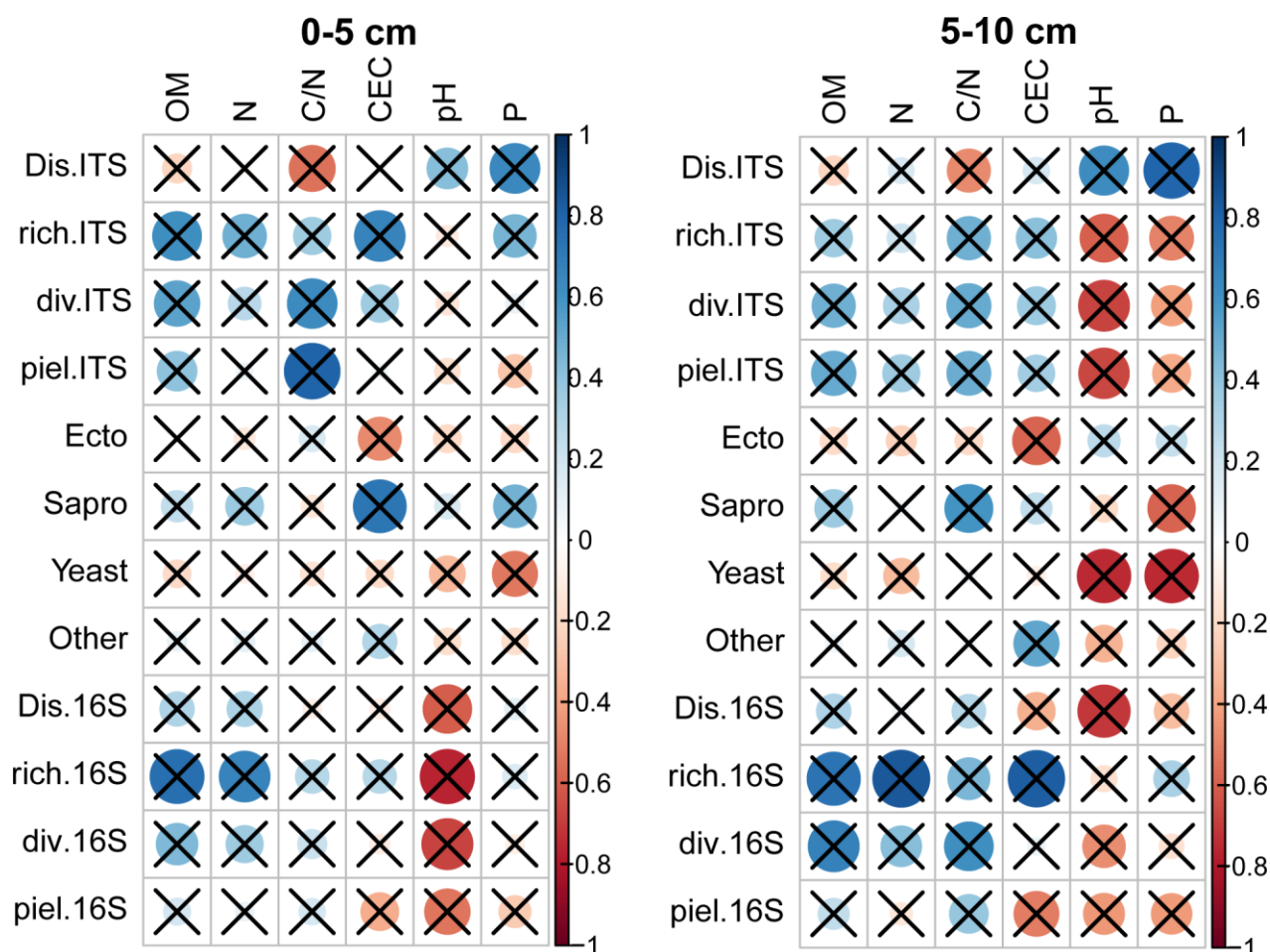
SUPPLEMENTARY FIGURE 2. SIMPER analysis identifying the % contribution of bacterial class representing at least 1% of contribution to the Bray-Curtis dissimilarity matrix between REF and OMR treatments in function of soil depth (0-5 and 5-10 cm) for the six studied sites.



SUPPLEMENTARY FIGURE 3. Impact of OM removal on fungal alpha-diversity for each of the six studied sites. OM impact on fungal species richness, Shannon index and Pielou's evenness were compared using two-way ANOVA and Tukey's HSD post hoc tests. Different letters indicate significant differences.



SUPPLEMENTARY FIGURE 4. SIMPER analysis identifying the % contribution of fungal genus representing at least 1% of contribution to the Bray-Curtis dissimilarity matrix between REF and OMR treatments in function of soil depth (0-5 and 5-10 cm) for the six studied sites.



SUPPLEMENTARY FIGURE 5. Picture of Pearson correlations between REF plots soil properties and the following measurements: i) bacterial and fungal alpha diversity between OMR and REF plots (\log_2 fold change OMR/REF), ii) bacterial and fungal dissimilarity between OMR and REF plots iii) fungal guilds relative abundance between OMR and REF plots (\log_2 fold change OMR/REF). Abbreviations: N, total nitrogen; OM, soil organic matter; P, organic phosphorus; Dis.ITS: dissimilarity index of fungal OTUs composition (OMR vs REF) calculated for each site; Dis.16S: dissimilarity index of bacterial OTUs composition (OMR vs REF) calculated for each site site.

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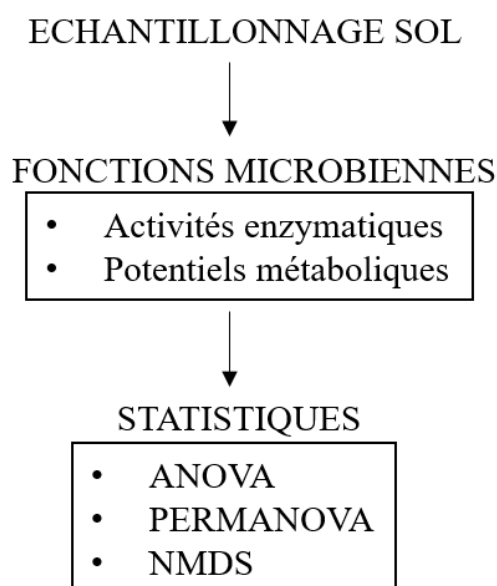
CHAPITRE III

Enzymatic activities and community-level physiological profiles (CLPP) in subsoil layers are altered by harvest residue management practices in a tropical *Eucalyptus grandis* plantation

Article de recherche publié dans la revue Microbial Ecology le 29 novembre 2018.

INTRODUCTION AU CHAPITRE III

Le chapitre III porte sur l'étude des fonctions microbiennes en réponse à la récolte d'arbre entier en plantation d'*Eucalyptus grandis* en climat tropical. Les fonctions microbiennes des sols de placettes d'Eucalyptus ayant deux modes de récoltes contrastés (récolte d'arbres entiers et récolte des tiges uniquement) ont été comparées par des approches enzymatiques (potentiel de mobilisation de certains éléments du sol) et d'évaluation des capacités de métabolisation de substrats carbonés (potentiel des activités cataboliques microbiennes). Les fonctions microbiennes ont été étudiées à trois profondeurs : 0-5, 5-10 et 10-20 cm.



**Enzymatic activities and community-level physiological profiles (CLPP) in
subsoil layers are altered by harvest residue management practices in a tropical
Eucalyptus grandis plantation**

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ABSTRACT

Harvest residue management is a key issue for the sustainability of *Eucalyptus* plantations established on poor soils. Soil microbial communities contribute to soil fertility by the decomposition of the organic matter (OM), but little is known about the effect of whole-tree harvesting (WTH) in comparison to stem only harvesting (SOH) on soil microbial functional diversity in *Eucalyptus* plantations. We studied the effects of harvest residue management (branches, leaves, bark) of *Eucalyptus grandis* trees on soil enzymatic activities and community-level physiological profiles in a Brazilian plantation. We measured soil microbial enzymatic activities involved in OM decomposition and we compared the community level physiological profiles (CLPP) of the soil microbes in WTH and SOH plots. WTH decreased enzyme activities and catabolic potential of the soil microbial community. Furthermore, these negative effects on soil functional diversity were mainly observed below the 0-5 cm layer (5-10 and 10-20 cm), suggesting that WTH can be harmful to the soil health in these plantations.

Keywords: Tropical forest soil, forest residue management, *Eucalyptus grandis*, enzyme activities, CLPP.

Areas of planted forests are expanding in tropical regions contributing to supply the growing demand in wood and wood-derived products (Paquette and Messier, 2010). High amounts of nutrients exported with tree biomass harvested every 6-10 years rise the issue of the sustainability of *Eucalyptus* plantations (Achat et al., 2015a). Recent meta-analyses confirmed negative impacts of intensive forest management practices, such as whole-tree harvesting (WTH), on soil properties and tree productivity (Achat et al., 2015b; Wall, 2012). Nevertheless, in comparison to stem only harvesting (SOH), the effect of WTH on tree and soil properties remains in debate regarding *Eucalyptus* plantations. Indeed, independent studies have shown either negative impacts (Epron et al., 2015; Laclau et al., 2010; O'Connell, 2004; Versini et al., 2013) or no effect of whole-tree harvesting on tree growth and soil properties (Carneiro et al., 2009; de Dieu Nzila et al., 2002; Jones et al., 1999; Mendham et al., 2003, 2002). Changes in tree productivity and soil properties after residue management might result from disturbances of the decomposition processes in the soil. The link between soil organic matter (OM) and tree productivity is mainly due to the activity of soil microorganisms, which contribute through degradation mechanisms to supply plant-available nutrients (van der Heijden et al., 2008). Microorganisms deploy extracellular enzymes to break down organic compounds into shorter assimilable molecules and therefore they participate to carbon and other nutrient cycles in soil (Caldwell, 2005; Waldrop and Firestone, 2004; Wallenstein and Weintraub, 2008). In comparison to SOH, WTH practices might weaken these recycling key processes in soil by decreasing the availability of soil carbon and nutrients at planting, then tree growth and litter production.

A negative effect of WTH in *Eucalyptus* plantations on soil microbial biomass was shown in Australia by Mendham et al. (2002), but potential effect on soil microbial functions remains unknown. To understand how WTH influence soil microbial functions, we compared the effects of WTH and SOH forest practices on soil microbial functional diversity, in an experimental *Eucalyptus grandis* plantation in South East of Brazil. As most of previous studies focused on forest floor, or topsoil (0-5 cm), we chose to examine additionally whether residue management practices influence deeper soil layers (5-10 and 10-20 cm). First, we hypothesized that residue removal would alter the functional potential of the soil microbial community. We also hypothesized that the soil microbial functional diversity decreases in response to residue removal, with higher impacts in the topsoil than in subsoil.

The study site is an experimental plantation located at Itatinga, São Paulo state (23°17'S and 48°28'O). The native savannah was replaced by *Eucalyptus spp.* Soil OM and nutrient contents are low (red yellow Ferralsol). Our study was carried out 4 years after replanting *Eucalyptus grandis* trees. We compared two treatments with contrasting harvest residue management during the inter-rotation period. First at the end of the first rotation, *Eucalyptus* trees were cut and only the stems were harvested. Leaves, branches and stem tops were let for decomposition on the soil. We called this treatment stem only harvesting (SOH). In the second treatment, *Eucalyptus* trees were completely harvested including leaves, branches and stem tops. This treatment was called whole-tree harvesting (WTH) and was considered as a more intensive forestry practice than SOH due to the greater amount of tree biomass harvested. We collected soil samples in three plots for each WTH and SOH treatment in November 2016 at the Itatinga *Eucalyptus grandis* experimental plantation. Sampling was conducted at this time of the year when soil moisture was relatively elevated as it is the maximum of soil microbial activity period (Boerner et al., 2005; Kang et al., 2009). Each plot (24 x 16 m) comprised 81 trees in 9 rows of 9 trees, with a double row buffer separating the adjacent plots. Forest floor was removed before sampling and six soil cores (5 cm diameter) were withdrawn per plot: five on a diagonal transect through the 5*5 tree inner plots and one random inside this plot (Figure S1). Soil cores were divided in three layers: 0-5, 5-10 and 10-20 cm, sieved at 2 mm, homogenized and then stored in cold room (+ 4°C) until analyses. To study the microbial functional diversity, we applied enzymatic activities and CLPP approaches. Enzymatic activities approach mostly reveals a direct in situ measurement of fungi OM degradation ability while CLPP approach mainly highlights bacterial community carbon substrates metabolization potential which renders this two techniques complementary (Adamczyk et al., 2015; Lladó and Baldrian, 2017; Preston-Mafham et al., 2002; Saiya-Cork et al., 2002). First, we measured activities of six enzymes involved in soil OM degradation and mobilization of C, nitrogen (N) and phosphorus (P): β -glucosidase, cellobiohydrolase, xylosidase, glucuronidase, for polysaccharide degradation; N-acetylglucosaminidase for chitin decomposition and N mobilization; and acid phosphatase, as functional indicator of P mobilization. Protocol was adapted from Saiya-Cork et al. (2002). Briefly, 1 gram of soil (n=18 soil samples per treatment per depth) was weighted and added to 20ml of acetate buffer (pH=5, 28.82mL of 1M acetic acid, 273.3mL of 0.3M sodium acetate and adjusted to 1 L with distilled water) to give a final concentration of 1:20 w/v. These soil suspensions were incubated at 4°C for one night under agitation. For microplate assays, in each well of a clear flat-bottom 96-well plate (Sarstedt, Newton, NC, USA), 200 μ L of the soil suspension were added to 50 μ L of the respective substrate solution. After incubation at 25°C,

the plate was centrifuged at 4,500g for 5 minutes and 50µl of the clear solution was transferred in a black opaque 96-well microplate (Perkin-Elmer, Life Sciences) containing 50µl of stopping buffer (Tris 2.5 M pH 10–11) in each well. Measurements were carried out using a Victor3 microplate reader (Wallac Perkin-Elmer Life Sciences, Villebon-sur-Yvette, France) with an excitation wavelength of 360nm and an emission wavelength of 450nm. Enzymatic activity (EA) was expressed in pmol of methylumbelliferone formed per gram of dry soil per minute. Secondly, we pooled soil samples per plot and applied a physiological profile approach (CLPP) using BIOLOG EcoplatesTM on each composite soil (n=3 soil composites per treatments per depths) as described by Uroz et al. (2013). CLPP approach is based on the measurement of the ability of the microbial community to metabolize 31 different carbon sources. The OD 590 nm of the reference well was subtracted from the other wells containing carbon sources. These corrected absorbances were used for the subsequent analysis. Statistical analyses and data representations were performed using R software (R Core Team 2016). Soil enzymatic data were log transformed to ensure normality. Soil enzymatic and CLPP data were analyzed using nonmetric multidimensional scaling analysis (NMDS) and permutational multivariate analysis of variance (PERMANOVA) based on Euclidean distance matrix using Vegan package (Oksanen et al., 2013). Soil enzymatic activities and soil CLPP were compared using analysis of variance (Two-way ANOVA with depth and treatment as factor).

Nonmetric multidimensional scaling (NMDS) analysis of the soil extracellular enzymatic activities and the CLPP data show that SOH and WTH treatments were closely clustered for the organic soil layer (0-5 cm). By contrast, the two other soil layers (i.e. 5-10 cm and 10-20 cm) segregated in function of treatment (Figure 1a). PERMANOVA using Euclidean distance matrix confirms that soil depth ($P \leq 0.001$) and treatment ($P \leq 0.001$) significantly influenced the soil extracellular enzymatic activities (Figure 1b). Soil depth and treatment explained 50.6% and 12.7% of the variation in distances, respectively. This analysis confirms a treatment effect on CLPP profiles ($P = 0.024$), treatment explaining 11.4% of the total variance (Figure 1b). PERMANOVA shows that soil depth impacted significantly CLPP profiles ($P=0.016$). For both enzymatic and CLPP approaches, dissimilarity index increased with soil depth (Figure 1c), indicating that harvest management practices impacted more the deeper soil horizons studied here (i.e.5-10 cm and 10-20 cm) than the topsoil (i.e. 0-5 cm). The activities of acid phosphatase and cell wall degrading enzymes decrease from the 0-5 cm to the 10-20 cm layers (Figure 2). It is well known that soil potential enzyme activities generally decline with soil depth (Šnajdr et al., 2008; Stone et al., 2014; Taylor et al., 2002). In the upper soil layer (0-5cm), only the xylosidase activity is significantly influenced by harvest

management practices ($P \leq 0.05$), decreasing by 13.6% in WTH treatment in comparison to SOH treatment. In the 5-10 cm layer, cellobiohydrolase and glucuronidase activities were 32.3% and 25.5 % lower in WTH than in SOH, respectively. WTH treatment disrupted all enzyme activities in the 10-20 cm soil layer. Indeed, the six enzyme activities measured were significantly ($P < 0.05$) lower in WTH than in SOH in the 10-20 cm layer (decreasing by 78.2% for cellobiohydrolase; 35.9% for glucuronidase; 23.5% for xylosidase; 14.7% for N-acetylglucosaminidase; 12.5% for β -glucosidase and 10.3% for acid phosphatase). In the same way, if rate of substrate utilization based on CLPP approach appeared to be slightly impacted by harvest management practices in the topsoil (0-5 cm), overall substrate utilization decreased respectively by 16% and 15% in the 5-10 cm and 10-20 cm layers in the WTH plots by comparison with the SOH plots (Figure 3). This decrease of total microbial catabolic potential in WTH treatment was mainly explained by the following substrate: L-serine, alpha-D-lactose, D-cellobiose, glycyl-L-glutamic acid, D-erythritol and N-acetyl-D-glucosamine. Both enzymatic activities and CLPP approach revealed a negative impact of WTH by comparison with SOH respectively on the effective functions and the functional potential of the soil microbial community. Such decreases in soil functional diversity resulting from forest intensification practices, as whole-tree harvesting, have already been observed (Brant et al., 2006; Kotroc o et al., 2014; Weintraub et al., 2013). As soil OM degradation occurs mainly in the topsoil, only this horizon is commonly sampled to investigate soil microbial response to OM manipulation (Adamczyk et al., 2015; Cookson et al., 2008; Kim et al., 2018; Pisani et al., 2016; Smolander et al., 2008, 2010; Weintraub et al., 2013). In our study, the soil microbial response to harvest residue management was higher below 5 cm depth than in the topsoil. In a meta-analysis comparing WTH to SOH, Achat et al. (2015b) reported that soil properties of deep layers were more negatively impacted than in topsoil. Chen and Xu (2005), in a subtropical pine plantation, showed that microbial biomass was relatively more negatively affected in subsoil than in topsoil in response to WTH harvesting. Taken together, these results revealed that soil properties, microbial biomass and microbial functions might be more negatively impacted in subsoil layer than in topsoil layer in response to intensive forestry. Consequently, the monitoring of these parameters, only targeted in the topsoil layer, cannot be considered as complete indicators of possible impact of intensive forest management. Changed in microbial functions we measured in response to WTH could be due to the direct impact of intensive forestry on soil OM and nutrients content or indirect impact such as modification of soil compaction, moisture and temperature that we have now to explore and bridge with soil microbial functions (Han et al., 2009; Mendham et al., 2003; Proe et al., 2001).

In conclusion, we demonstrate that whole-tree harvesting negatively impacted the functional diversity of soil microbes in comparison to stem only harvesting, resulting in a decrease in microbial enzymatic activities involved in C, N and P cycling. Nevertheless, the functional gaps measured between these two treatments exhibited opposite responses to our prediction. Indeed, residue-removing effects were more pronounced in the deepest studied layers, providing a lasting imprint of intensive harvest management practices in subsoil horizons. Our findings highlight the need to explore the consequences of forest management on the functioning of the relatively deep soil horizon under tropical climate.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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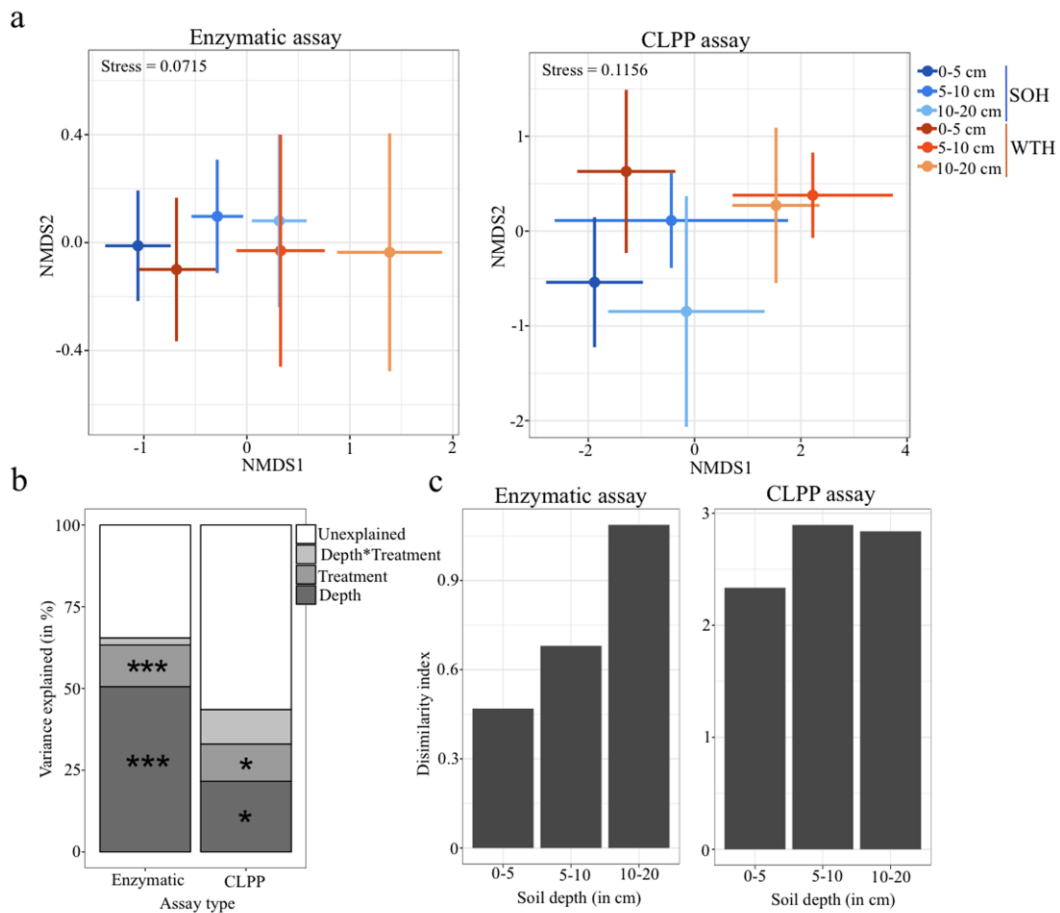


FIGURE 1. Figure 1. (a) First two axes for the nonmetric multidimensional scaling analysis (NMDS) of the six enzymes activities and the thirty-one substrates of the CLPP approach based on Euclidean distance dissimilarity matrix between groups (i.e. SOH 0-5 cm, SOH 5-10 cm, SOH 10-20 cm, WTH 0-5 cm, WTH 5-10 cm and WTH 10-20 cm). (b) Variances explanation based on permutational multivariate analysis of variance using Euclidean dissimilarity matrix for enzymatic and CLPP approaches ($P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.001$ ***). (c) Dissimilarity index calculated for the six enzymes activities and the thirty-one substrates of the CLPP approach based on Euclidean dissimilarity matrix between WTH and SOH treatments in function of depth (i.e. 0-5, 5-10 and 10-20 cm).

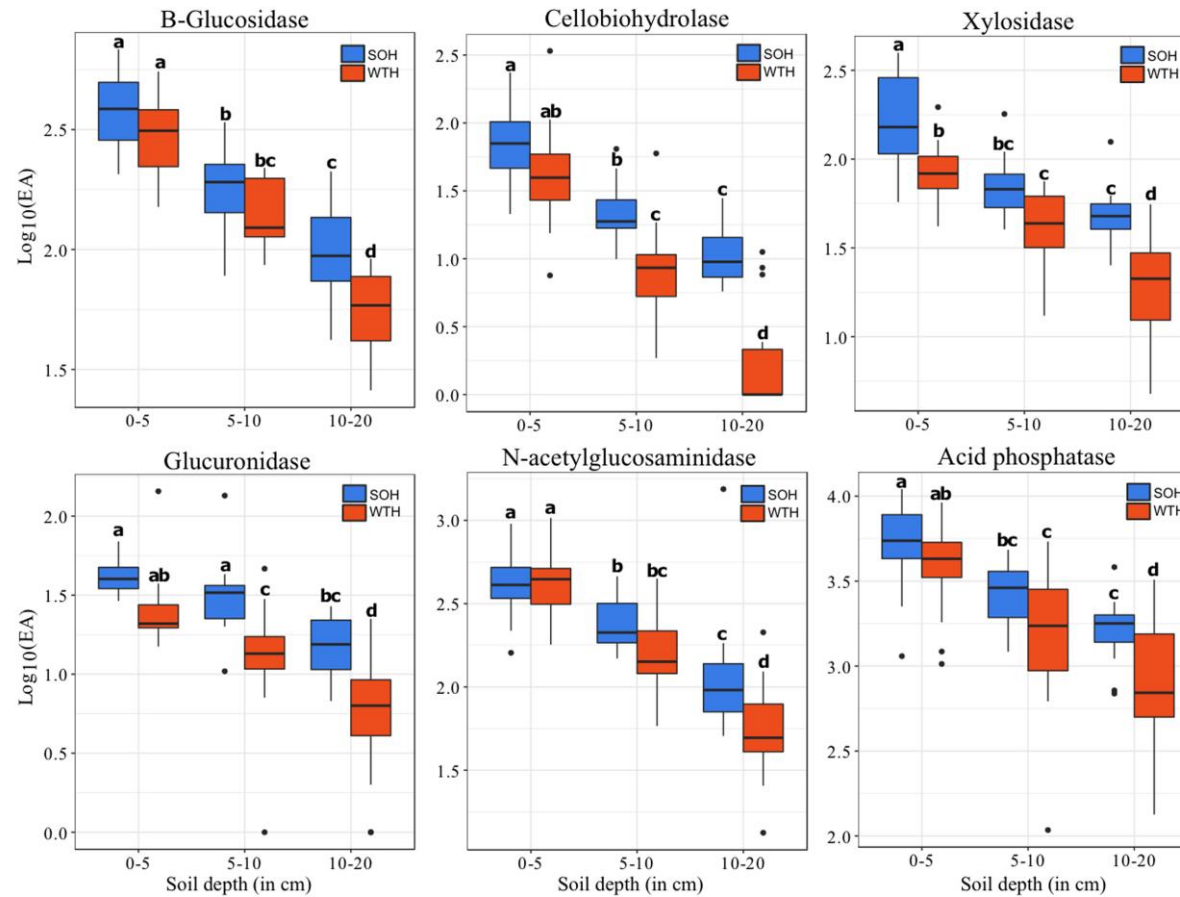


FIGURE 2. Soil enzyme activities (i.e. acid phosphatase, cellobiohydrolase, glucosidase, glucuronidase, N-acetylglucosaminidase and xylosidase) in function of depth (i.e. 0-5, 5-10 and 10-20 cm) and treatment (i.e. SOH and WTH). Different letters denote significant differences within each group ($p \leq 0.05$, Two-way ANOVA and Tukey's test, $n=18$).

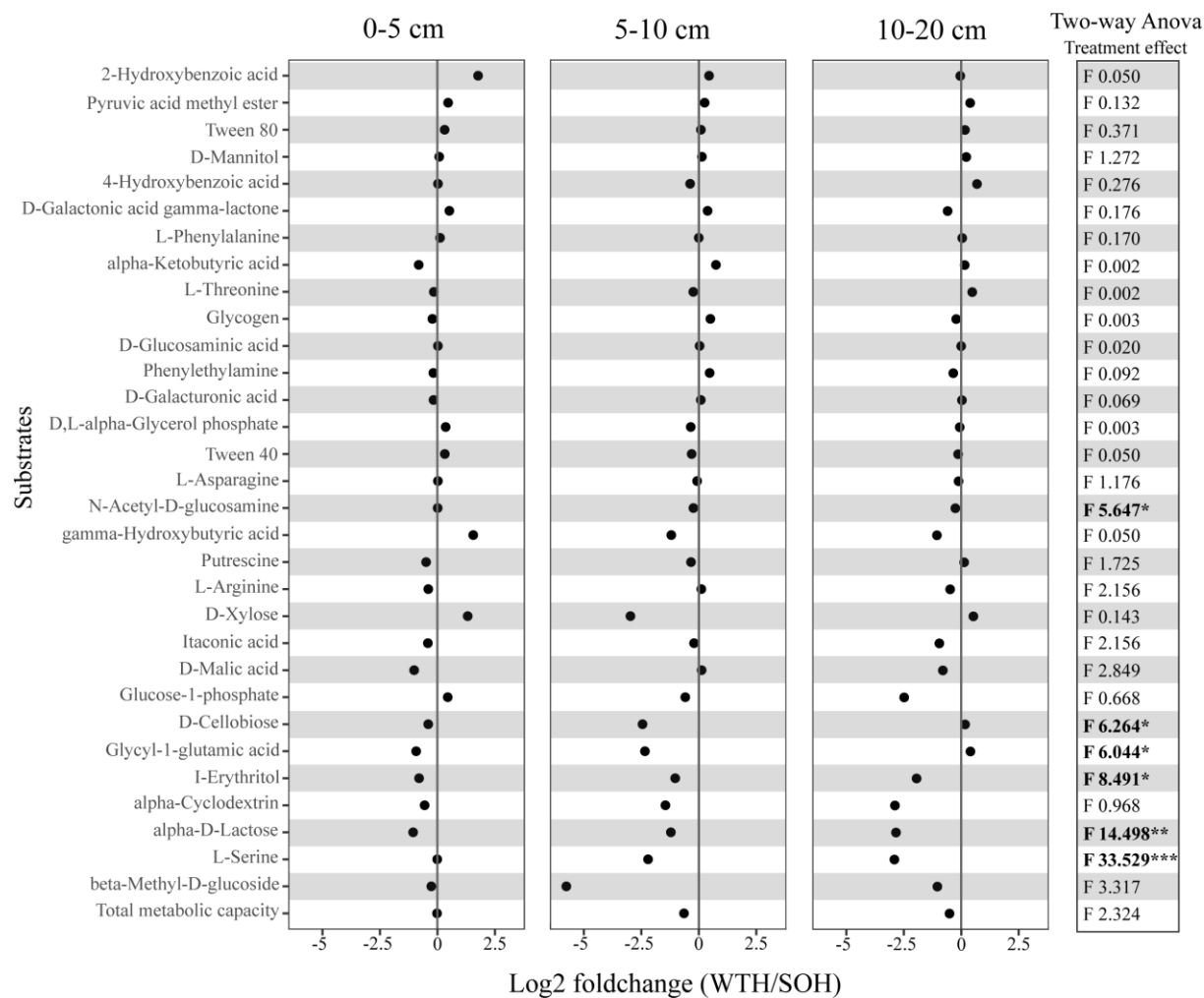


FIGURE 3. Effect of WTH on metabolization capacity of the 31 substrates measured with CLPP approach [\log_2 (fold change WTH over SOH)] in function of depth (i.e. 0-5, 5-10 and 10-20 cm). Differences in metabolization capacity was assessed using two-way ANOVA ($P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.001$ ***).

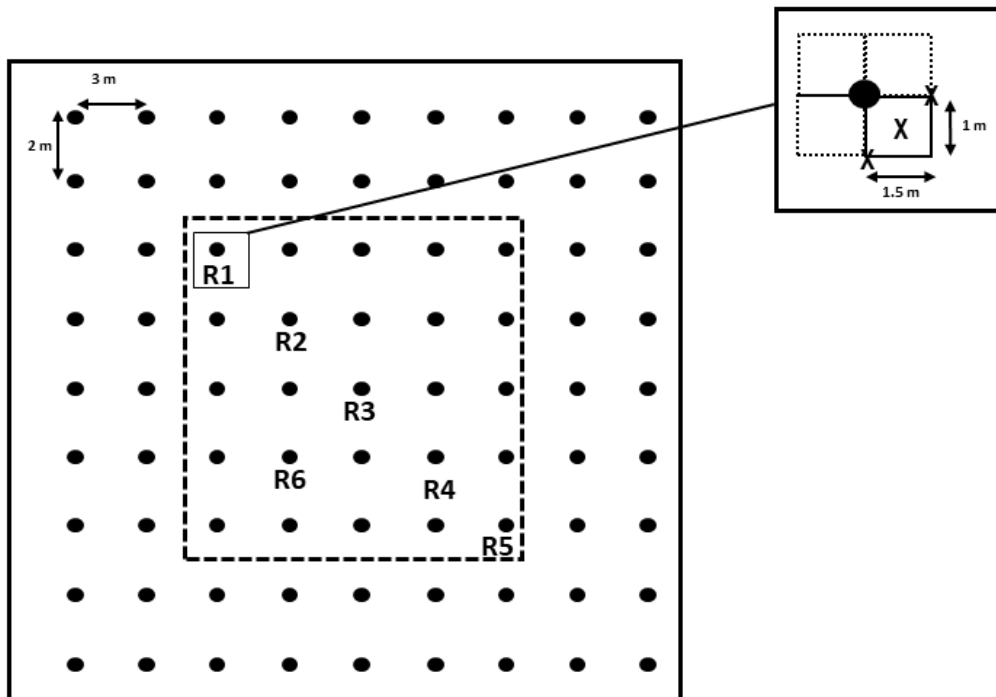


Figure S1. Example of sampling design for one plot. Black dots represent *Eucalyptus grandis* trees. R 1:6 represent the name of the samples for one plot. R6 sample position was randomly chosen in each plot. Cross represents the sampling zone near each *Eucalyptus grandis* tree.

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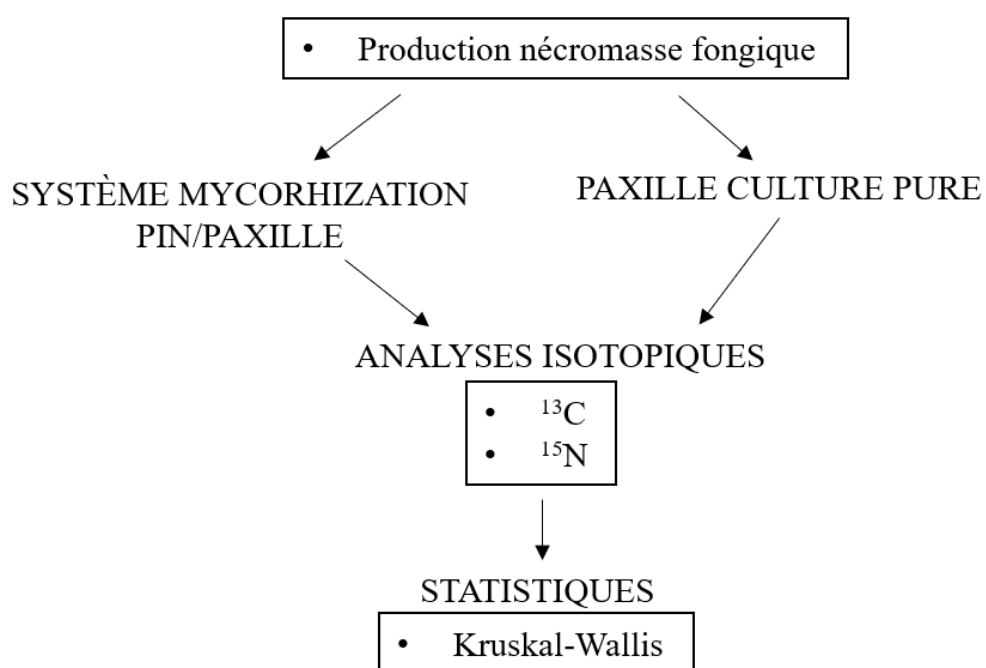
CHAPITRE IV

First evidences that the ectomycorrhizal fungus *Paxillus involutus* mobilizes nitrogen and carbon from saprophytic fungus necromass

Article de recherche publié le 11 octobre 2018 dans la revue Environmental Microbiology.

INTRODUCTION AU CHAPITRE IV

Le chapitre IV concerne l'étude des capacités de dégradation de la nécromasse fongique par *Paxillus involutus* en condition d'interaction symbiotique avec le Pin et en culture pure. Cette étude a été conduite en microcosme en condition monoxénique. Nous avons produit de la nécromasse fongique (*Postia placenta*) enrichie en ^{15}N et ^{13}C . Le carbone et l'azote marqués provenant de cette nécromasse fongique et mobilisés par *Paxillus involutus* ont ainsi été suivis dans la plante, dans les ectomycorrhizes et dans le mycélium du champignon mycorhizien.



First evidences that the ectomycorrhizal fungus *Paxillus involutus* mobilizes nitrogen and carbon from saprophytic fungus necromass

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Running title: Do ectomycorrhizal fungi degrade brown rot fungi?

Summary

Fungal succession in rotting wood shows a surprising abundance of ectomycorrhizal (EM) fungi during the late decomposition stages. To better understand the links between EM fungi and decay fungi, we investigated the potential capacities of the EM fungus *Paxillus involutus* to mobilize nutrients from necromass of *Postia placenta*, a wood rot fungus, and to transfer these elements to its host-tree. In this aim, we used pure-cultures of *P. involutus* in presence of labelled *Postia* necromass ($^{15}\text{N}/^{13}\text{C}$) as nutrient source, and a monoxenic mycorrhized pine experiment also, composed of labelled *Postia* necromass and *P. involutus* culture in interaction with pine seedlings. The isotopic labelling was measured in both experiments. In pure culture, *P. involutus* was able to mobilize N, but C as well, from the *Postia* necromass. In symbiotic interaction experiment, we measured high ^{15}N enrichments in all plant and fungal compartments. Interestingly, ^{13}C remains mainly in the mycelium and mycorrhizas, demonstrating that the EM fungus transferred essentially N from the necromass to the tree. These observations reveal that fungal organic matter could represent a significant N source for EM fungi and trees, and C source also for mycorrhizal fungi, suggesting that the fungal partner controls carbon market in EM symbiosis.

Introduction

In forest ecosystems, dead wood and soil organic matter (SOM) represent essential habitats for a wide variety of organisms (Kruys and Jonsson, 1999; Nordén et al., 2004) and consequently an energetic and biological motor for the associated ecosystem services, such as soil carbon sequestration (Clemmensen et al. 2013). Interestingly, if soil microorganisms are highly involved in organic matter decomposition and cycling, their own cell degeneration and their degradation products are important components of this SOM (Schmidt et al. 2011). Indeed, plant tissues, soil fauna and microorganisms are the primary sources of soil organic carbon in forest soils (Six et al. 2006).

In these ecosystems, saproxylic fungi are highly relevant in the process of the degradation of dead wood to mobilize carbon (Blanchette, 1995; Rajala et al. 2015) and recycle the other major nutrients, such as nitrogen and phosphorus (Watkinson et al. 2006). In addition, this decomposition of woody debris is a temporal dynamic process supported by the succession of different fungal ecological groups (Stockland et al., 2012). Interestingly, diverse studies reported the recurrent presence and potential role of ectomycorrhizal (EM) fungi in this decaying process or in the saproxylic food web dynamics (Tedersoo et al. 2003; Buée et al., 2007; Rajala et al., 2012; 2015; Walker et al. 2012). EM fungi are well known to provide soil-derived nutrients in exchange for carbohydrates produced by photosynthesis from the host tree. This exchange of commodities between two interacting partners is the most basic element of any biological market (Werner et al., 2014). In the EM symbiosis this market involve the exchange of different commodities by both partners. Indeed, EM fungi are highly efficient at exploring the soil and mobilize nutrients, particularly organic ones that would not be directly available to their plant hosts, their carbon suppliers (Smith & Read, 2008). Therefore, rotting wood could constitute a particular niche for EM fungi involved in a symbiotic interaction with their host-tree roots. More precisely, it was suggested that certain EM fungi colonized dead wood acting as scavengers by degrading wood rot fungi (Buée et al. 2007; Rajala et al., 2012; Mäkipää et al. 2017; Maillard et al., 2018). The abilities of EM fungi to mobilize nutrients from dead organic matter have been suggested in numerous studies (Buée et al., 2005; Courty et al., 2007; Lindahl & Finlay, 2006; Talbot et al., 2013; Vaario et al. 2002). Some authors have even reported that EM fungi would be able to mobilize carbon from plant organic matter (e.g. cellulose and hemicellulose) for their development (Durall et al., 1994); but also, under certain conditions, to mitigate or offset losses in the host-tree carbon budget through a mixotrophic interaction (Cullings and Courty, 2009; Bréda et al., 2013). Rineau et al. (2012) have

investigated the mechanisms by which *Paxillus involutus* could degrade organic matter. The expression profile of genes potentially involved in organic matter degradation revealed that only few of them were expected to be involved in plant cell wall degradation. Additionally, Rineau et al. (2012) reported in this study that *P. involutus* induces chemical changes in plant organic matter similar to hydroxyl radical attacks of brown-rot fungi, involving Fenton chemistry. Furthermore, more recently Kohler et al. (2015) demonstrated strong losses of genes encoding enzymes involved in decay of lignin within EM fungal genomes, including class II peroxidases (PODs) or glyoxal oxidase. In short, although some EM fungi have retained a reduced repertoire of plant cell wall-degrading enzymes (PCWDEs), they have limited genomic capacity to degrade lignocellulose. Nevertheless, thanks different strategies, EM fungi could improve organic matter decomposition and co-metabolic degradation of recalcitrant organic complexes, thereby releasing the organic N necessary to their hosts (Lindahl and Tunlid, 2015).

While numerous studies have focused their attentions on plant organic matter and deciphered microbial lignocellulolytic functions, other papers increased focus on the decomposition of microbial organic matter. In particular, fungal residues provide polymeric substances, such as chitin, glucans, lipids, melanin and wide range of proteins, corresponding to diverse sources of nutrients in soils (Bartnicki-Garcia 1968; Kögel-Knabner, 2002; Godbold et al., 2006; Lorenz et al. 2007; Nannipieri and Eldor, 2009). As an example, soil chitin is derived mainly from fungal and arthropod necromass, and even if chitin is the most abundant aminopolysaccharide in nature (Kumar, 2000), little is known about the turn-over of this large pool of organic nitrogen. Different authors have suggested that EM fungi are capable of degrade fungal necromass and to lyse associated cell wall. Indeed, Leake and Read (1990) have reported potential chitinolytic activities from EM fungi by quantifying the mycelial growth of diverse ericoid and EM fungi with chitin as the sole source of nitrogen. Complementary to these observations, enzymatic approaches confirmed the expression of proteolytic and chitinolytic activities from EM fungal mycelium in presence to saprophytic fungal necromass, or pure chitin (Mucha et al., 2006; 2007; Maillard et al. 2018). Moreover, Lindahl and Taylor (2004) reported the frequent occurrence of N-acetylhexosaminidase-encoding genes in ectomycorrhizal fungi. Finally, although some studies have shown EM chitinase or proteolytic activities in presence of fungal necromass, there has been no effective demonstration of nitrogen and carbon mobilization from fungal cell wall to EM fungus, with a potential transfer to the plant host.

The use of biomarkers in combination with stable isotope analysis is an efficient approach in microbial ecology to study carbon or nitrogen transfer. Coupled with analytical techniques, such as gas chromatography-combustion-isotope ratio mass spectrometry, stable isotope labelling (e.g., for ^{15}N uptake or ^{13}C mobilization or respiration) provides the unique opportunity to link biological identity (biomarker) and biopolymer decomposition and associated activities (Boschker and Middelburg, 2002). Using $^{13}\text{C} / ^{15}\text{N}$ stable isotopic tracing, the aims of our study were i) to prove the capability of an EM fungus to decompose the necromass of lignolytic fungus and mobilize nutrients (N and/or C) in pure-culture experiment; ii) to extend this demonstration to symbiotic conditions; iii) to quantify N, and potentially C, transfers from the fungal necromass to the EM fungus and its host-plant in a symbiotic lifestyle of both partners and iv) to propose an allocation model of C and N in this symbiotic association connected to fungal necromass, as major nutrient resource. Finally, this study could provide founding results to prove the EM fungi role in the decomposition of microbial necromass and to better understand the C and N loop closing in forest ecosystems. In that order, we traced the flow of labelled fungal necromass in two controlled systems: pure-culture of the EM fungus *P. involutus* and tree seedlings inoculated with this fungus in monoxenic conditions.

Results

$^{13}\text{C}/^{15}\text{N}$ isotopic and enzymatic activity measurements in EM pure-culture experiment

First, we measured the direct incorporation of ^{15}N and ^{13}C in *Pa involutus* mycelium growing in presence of *P. placenta* labelled necromass. The isotope analyses were also conducted on dead mycelium of *P. involutus* for the quantification of residual labelling after washing. Thus, the transfers of ^{13}C and ^{15}N isotopes were assessed by calculating both enrichments within alive and freeze-dried *P. involutus* mycelium (Table 1). After 20 days of pure culture at 20°C in the dark, we measured significant ^{13}C and ^{15}N enrichment in *P. involutus* mycelium growing in the presence of labelled necromass in comparison with the control and unlabelled treatments ($P < 0.001$). The $\delta^{13}\text{C}$ have shifted from -12.68‰ in unlabelled necromass conditions to 123.50‰ in labelled necromass conditions, and $\delta^{15}\text{N}$ have shifted from 8.77‰ in unlabelled necromass conditions to 947.66‰ in labelled necromass conditions. Freeze-dried *P. involutus* mycelium (patches) was slightly enriched in the presence of labelled necromass (adsorbed material). But this residual labelling remained very low, approximately 3% of the ^{13}C and ^{15}N living *P. involutus* labelling.

From the same pure-culture experiments, soluble protein contents of washed *P. placenta* necromass were quantified as relatively low (1,2 % of dry mass). Moreover, *P. placenta* necromass was hydrolysed also to determine chitin content. Because we found that chitin represented 15,3% of *P. placenta* dry mass (details in Experimental Procedures), we measured the N-acetylglucosaminidase activity of *P. involutus* in presence of this necromass. The N-acetylglucosaminidase activity measured in the culture medium of *P. involutus* was significantly higher (pval<0.05) in the presence of unlabelled or labelled saprotrophic fungal necromass than in the control treatment, without necromass (Figure S3). Indeed, the constitutive N-acetylglucosaminidase activity of *P. involutus* was close to 400 pmol/ml/min⁻¹ in the absence of necromass. This enzymatic activity was increased by 50% in the presence of *P. placenta* necromass. These increases were similar with unlabelled and labelled necromass. We did not detect N-acetylglucosaminidase activity from solution containing only the *P. placenta* necromass, confirming that the extracellular enzymatic activity measured in our experiments was exclusively due to *P. involutus*.

¹³C/¹⁵N isotopic results for the ectomycorrhized Pinus seedlings experiment

In a second experiment, we measured the transfer of labelled elements in an experimental device consisting of *P. involutus* and *Pinus sylvestris* in presence of labelled, or unlabelled, necromass. The ¹³C and ¹⁵N labelling was assessed by calculating $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ for each biological compartment of symbiotic partners: EM mycelium, EM root tip, stem and needle (Table 2). Furthermore, we separated necromass plugs from perlite / mycelium mixture for residual labelling measures at the end of the experiment. Indeed, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were quantified from agar patches containing the lignolytic fungi necromass before and after consumption by the ectomycorrhizal fungus. On the other hand, since it was technically difficult to separate the *P. involutus* extraradical mycelium from the perlite substrate, they were considered to be a single compartment. The differences of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ between natural abundance and labelled conditions were significant for all compartments. Moreover, as a control, we quantified C and N from pure sterile perlite using continuous flow CN analyser (Carlo Erba NA1500). We measured negligible quantities of C and N (0.03% and 0.006% respectively), confirming that the C and N measured in the mycelium/perlite compartment belonged only to the fungal material and/or root exudates.

In comparison to the control, the ¹⁵N enrichment was highly significant for all tree compartments and for the EM mycelium colonizing the perlite. The mycorrhized roots were the

most enriched compartments. In addition, the mycorrhized roots and the ectomycorrhizal mycelium were highly enriched in ^{13}C (Table 2), but the $\delta^{13}\text{C}$ decreased progressively from the EM fungal mycelium to the stem, with a relatively low labelling in the needles. Indeed, in the plant aboveground compartments (stem and needles, on average), the $\delta^{13}\text{C}$ changed from -24.3‰ for the control to -18.5‰ for the labelled treatment, and, in the belowground area, the $\delta^{13}\text{C}$ shift from -12.3‰ to +2.1‰ (EM mycelium in perlite).

In the supplemental conditions, where two Magenta boxes were connected (Figure S1), the non-mycorrhizal control plants, without necromass, were not enriched in ^{15}N and ^{13}C , and the δ ‰ in the needles were similar to the values measured from the needles of control plants growing with unlabelled necromass: $\delta^{13}\text{C} = -27.65$ ‰ and $\delta^{15}\text{N} = -7.99$ ‰. These results demonstrated the absence of enrichment by gas exchanges, in particular CO_2 resulting from EM fungal respiration and C mineralization.

Allocation model of the enrichment by biological compartment in the symbiotic association

The quantities (mg) of ^{15}N and ^{13}C were calculated by compartment for each tree from the compartment biomass and the value measured in $^{13}\text{C}/^{15}\text{N}$ atomic percent. Nevertheless, it was not possible to separate the *P. involutus* mycelium from perlite, and therefore to quantify the biomass corresponding to this extramatricial mycelium. In this case only, we used δ ‰ values because the very low biomass of *P. involutus* was negligible compared to the pine biomass: roots, stems and needles. The quantities of carbon and nitrogen by compartment were compared between the two treatments: labelled and unlabelled necromass patches. The proportions of ^{15}N and ^{13}C and their distribution in the different biological compartments were estimated from the labelled fraction that had been mobilized from the necromass patches in comparison to initial labelling, as illustrated in Figure 2. Almost 10,9% of ^{13}C and 52,5% of ^{15}N was mobilized from labelled necromass patches. Over 90% of this mobilized ^{15}N was transferred to the tree compartments: EM roots, stem and needles. In contrast, less than 3% of the ^{13}C (about 27% of mobilized ^{13}C) was located in the aboveground part of the tree. Interestingly, in this $^{13}\text{C} / ^{15}\text{N}$ balance model, more than 72.5% of mobilized ^{13}C was located in roots mycorrhized by *P. involutus* and in its extramatricial mycelium.

Discussion

The communities of ectomycorrhizal fungi play a major role in plant nutrition and, in recent studies, this fungal ecological group has been described as a significant input into forest carbon pools (Read and Perez-Moreno, 2003; Fernandez et al. 2016). Because EM fungi constitute this important carbon pool in the soil (Clemmensen et al., 2013), the majority of studies have investigated the role of bacteria, or saprophytic fungi, in the decomposition of the EM fungal necromass (Drigo et al., 2012; Fernandez and Koide, 2012; Russell, 2014; Fernandez et al. 2016). However, there is little evidence on the potential involvement of EM fungi in the degradation of soil necromass compounds, especially fungal or insect cell walls and proteins (Klironomos and Hart, 2001; Mucha et al. 2006). In our experiments, we used fungal necromass of *P. placenta* as the sole form of organic material (Di Mario et al. 2008; Hartl et al., 2012; Fernandez and Koide, 2012).

Arising from labelled fungal necromass decomposition, our results demonstrated that *P. involutus* is able to partially decompose the necromass of a lignolytic fungus and to mobilize N and C from this organic matter. Because the necromass was rinsed several times in demineralized water before freeze-drying, we assume that the cells of the brown rot fungus were largely emptied of their cytoplasmic contents and soluble amino acids, disaccharides and monosaccharides. In the present study, chitin content in *P. placenta* necromass was slightly above the amount given in the literature for Basidiomycota species, which is comprise between 3% and 11% (Plassard et al. 1982, Fernandez and Koide, 2012). Therefore, N-acetylglucosaminidase activities suggest that *P. involutus* could decompose chitin polymers and associated melanin macromolecules of the cell wall of *P. placenta*, although we cannot rule out that a significant portion of labelled C / N was mobilized from lipids, nucleic acids or residual proteins. Subsequent studies, coupling Gas Chromatography or Liquid Chromatography with IRMS could be conducted to identify the different classes of molecules mobilized by the EM fungus in presence of fungal necromass. In a previous study, Leake and Read (1990) compared the capacity of ericoid and ectomycorrhizal species to degrade crystalline α -chitin, suggesting the ability of *P. involutus* to use this crustacean's chitin as a source of nitrogen. Nevertheless, *P. involutus* was much less effective at degrading chitin than ericoid fungi (Leake and Read, 1990), or than EM Agaricales species (Maillard et al. 2018). Nevertheless, a great variation of chitinases related to nutritional chitin acquisition, competitive interaction, autolysis or cell wall remodeling can be expressed in natural conditions (Kellner and Vandenbol, 2010; Langner and Göhre, 2016). For this reason, only future transcriptomic or proteomic approaches could

provide a holistic view of mechanisms involved in fungal necromass degradation, as reported for plant cell wall (Shah et al. 2016). Moreover, *P. involutus* and other EM fungi can also depolymerize proteins and assimilate the released N from various organic matter sources (Shah et al., 2013). Consequently, use of labelled pure chitin in future studies would allow to prove the degradation of fungal chitin polymers by *P. involutus* and quantify the proportions of C and N potentially extracted from this polymer. This potential capacity should be checked also for other EM fungi, since some mycorrhizal groups probably have limited capacity to transform organic matter in comparison to certain lineages (Lindahl and Tunlid, 2015; Maillard et al. 2018).

Interestingly, when the EM fungus is in interaction with the host plant, it is still capable of mobilizing nitrogen but also carbon from the *Postia* necromass. Indeed, the strong accumulation of ^{15}N , especially in the aerial compartments, demonstrated significant transfer of N from fungal necromass to the plant through the EM fungus. In addition, ^{15}N labelling distribution in the plant revealed that the needles were a more relevant N sink than the roots. Although the EM fungus mainly mines N from *P. placenta* necromass, it is also able to mobilize C. In contrast, the enrichment in ^{13}C in the host tree was relatively weak, even though the labelling was significant in the needles. Because we have experimentally controlled that this C labelling was not the result of *P. involutus* respiration (CO_2 production), these low amounts of fungal C could come from amino acids or amino sugars, as glutamine. Indeed, Finlay et al. (1988) reported that several EM fungi were able to assimilate ^{15}N -labelled glutamate/glutamine, with a relatively low supplying to the host plant. Nevertheless, in this study, *P. involutus* was the only species unable to assimilate these N sources. In pure-culture, the significant proportion of ^{13}C measured in the *P. involutus* mycelium revealed for the first time that the EM fungus could mobilize carbon from the *P. placenta* necromass and accumulate it in its mycelium. This original result was supported in the monoxenic symbiotic experiment, by the $\delta^{13}\text{C}$ measurements in the perlite/EM extramatricial mycelium mixture and in pine mycorrhiza that were significantly higher in the samples collected in the labelled condition compared to the “natural abundance” condition. This experiment reveals that *P. involutus* is able to assimilate C from fungal necromass and integrate it preferentially in its own metabolism with low transfer to the host-tree. As suggested by Fellbaum et al. (2014), our study demonstrates how the EM fungus is able to control the interaction, despite being dependent on its host. When the EM fungus is interacting with its host, photosynthetic carbon availability must trigger the decomposition of the fungal organic matter enriched in N and its assimilation by the symbiotic

fungus. However, although EM fungus mainly mobilizes N from this organic matter, it also exploits some of the C available in the necromass. Finally, while this mutualistic symbiosis based on the reciprocal exchange of tree photosynthetic carbon for fungal-acquired nutrients from organic matter, the fungal partner could compensate for the energetic ‘cost’ resulting from the degradation of proteins and polymers enriched in N, due to the C mobilization that results from this decomposition.

A previous study showed that *P. involutus* induced chemical changes in organic compounds from plant material but it would not be able to assimilate released carbon in the presence of polysaccharides and complex cellulolytic compounds (Rineau et al., 2012). In our study, we demonstrated that *P. involutus* was able to assimilate not only N but also C from the fungal necromass of a brown rot fungus (*P. placenta*). Moreover, our experiment highlighted a relevant mobilization of nitrogen and an important transfer towards the trees in symbiotic conditions. These results were obtained in absence of carbon starvation, since the plant host had active photosynthesis. Indeed, it has recently been reported that the C availability directly drives the nitrogen mobilization of *P. involutus* as well as its activities of litter degradation, but in C-limiting conditions (Rineau et al., 2013). Carbon starvation could induce transcription of the genes encoding laccases, chitinase, peptidases and the transcripts of N-transporters.

In natural conditions, plant phenology, seasonal carbon reserve dynamics and photosynthetic cycles (winter/summer) regulate the dynamics of soil organic matter decomposition by EM fungi and the associated mobilization of N and possibly C under certain conditions (Buée et al., 2005; Talbot et al., 2008; Žifčáková et al., 2016). Interestingly, the role of EM fungi in fungal necromass degradation could explain the relative abundance of certain EM fungal species in decayed wood, where they can represent up to 80% of the fungal community identified during the final phase of the decay process (Rajala et al., 2012; 2015; Angst et al., 2018). Furthermore, these hypotheses are in line with the high chitinase activities measured from the EM root tips sampled in dead woody debris colonized by lignolytic fungi (Buée et al. 2007). These “scavengers” would act at the end of the degradation process of organic matter in the forest. Finally, the hypothetical fluxes of C and N between soil organic matter and plant and fungal components of forest ecosystems should be revisited including non-plant organic matter (Lindahl and Tunlid, 2015). Indeed, EM fungi mobilize N from the soil organic matter pool, thanks to oxidative mechanisms, Mn-peroxidases or Fenton reactions, and even if the metabolic C demand of these symbiotic fungi is not met by organic matter decomposition, some of them could still mobilize C from microbe and soil fauna necromass. Additionally, it is well known

that wood N content increases during the decay process (Watkinson et al. 2006; Rajala et al. 2012; Rinne et al. 2017). This accumulation rate of N at the end of wood degradation would promote the development of EM fungi, but also the establishment of competition with saprophytic fungi. Increase in the relative abundance of EM fungi in these late stage of decay could decreased decomposition of wood by alleviating nitrogen limitation of saprotrophs—as a “Gadgil effect” (Gadgil and Gadgil, 1971). Coupled with recent observations (Mäkipää et al. 2017; Maillard et al. 2018), our results illustrate the complexity of trophic networks in forest soil and microbial competitions for nutrient mobilization. The coupling of controlled studies, targeting other EM fungi, with field observations may be helpful in future investigations. It will be necessary to demonstrate the fungal necromass degradation ability and the ecological role of specific wood-inhabiting EM fungi, particularly the dominant genera in late stages of degradation, such as *Tylospora*, *Piloderma*, *Lactarius*, *Russula* or *Tomentella* (Buée et al. 2007; Rajala et al. 2012; Mäkipää et al. 2017). The goal of these future studies will be to bridge the current gap between genetic studies in the laboratory, in particular on genes and molecular processes involved in microbial cell wall degradation and mechanisms of speciation for these specific EM species, and field studies on interactions between organisms in their natural. Taking the role of EM fungi in microbial necromass degradation into consideration, we should improve our understanding of the role of EM fungi in forest C and N cycles, and modulate the paradigm of mycorrhizal symbiosis by revisiting the C organic market in this plant-fungus interaction.

Experimental Procedures

Experimental design

In short, nutrient transfers were traced by ^{15}N and ^{13}C enrichment of the saprotrophic fungal necromass. Two separate experiments were carried out. In the first experiment, *Paxillus involutus* (strain Pi 01) was grown in sterilized pure liquid culture enriched with or without the necromass of a saprotrophic fungus (*P. placenta*, strain PP978/9). We measured the ^{15}N and ^{13}C enrichment in *P. involutus* and the exo-chitinolytic activities (N-acetylglucosaminidase) of this EM fungal model. In the second experiment, we used a monoxenic system with tree seedlings inoculated with *P. involutus* in the presence or absence of *P. placenta* necromass. The transfers of ^{15}N and ^{13}C from the saprotroph necromass were measured in above- and belowground mycorrhized plant compartments including root tips and extramatricial mycelium in the mineral substrate (perlite). This monoxenic dual culture was maintained in plastic

Magenta boxes, previously autoclave sterilized. A control was added to measure the potential exchange of $^{13}\text{CO}_2$ within the Magenta box atmosphere (Figure S1). Both experimental ways are explained in detail in the following sections.

Biological resources and *P. placenta* necromass production

Pure cultures of fungal material: Paxillus involutus and Postia placenta

In sterile conditions, the EM fungus *Paxillus involutus* was cultured in Petri dishes at 20°C in the dark on modified Melin-Norkrans (MMN) medium (Marx, 1969), without malt extract: 10 ml.l⁻¹ KH₂PO₄, (NH₄)₂HPO₄, CaCl₂ (H₂O)₂, NaCl, MgSO₄ (H₂O)₂, thiamine (100 µl.l⁻¹), FeCl₃·6(H₂O) (100 µl.l⁻¹), glucose (2.5 g.l⁻¹), agar (15 g.l⁻¹), pH 5.5. The brown-rot fungus *P. placenta* was cultured in liquid medium comprised of 7 g.l⁻¹ glucose, 3 g.l⁻¹ (NH₄)₂SO₄, 2 g.l⁻¹ of KH₂PO₄, 0.4 g.l⁻¹ of MgSO₄·7(H₂O), 1mg.l⁻¹ Thiamine HCl and 1 mL of microelement solution adjusted to pH 5, then autoclaved. The microelement solution contained: MnCl₂ (H₂O)₄ (6 mg.l⁻¹), H₃BO₃ (1.5 mg.l⁻¹), ZnSO₄·7(H₂O) (2.65 g.l⁻¹), KCl (0.75 g.l⁻¹), Na₂MoO₄·2(H₂O) (0.0024 mg.l⁻¹), CuSO₄·5(H₂O) (0.13 mg.l⁻¹).

For the labelled / unlabelled biomass production of *P. placenta*, the fungus has grown in sterile conditions during three months in Erlenmeyer flasks (500ml), providing about 1 g.l⁻¹ of dry weight after filtration. *P. placenta* was selected because the EM fungus *P. involutus* expressed higher N-acetylglucosaminidase activity in the presence of the *P. placenta* necromass during a previous screening comparing 12 different lignolytic fungal strains: six brown rot fungi and six white rot fungi (data not shown). Moreover, as result of a pilot enrichment study focusing on a ¹⁵N and ¹³C labelling range of *P. placenta*, the fungus was cultivated in the presence of ammonium enriched with ¹⁵N and ¹³C glucose (the first carbon of the glucose was labelled) to obtain a final isotopic abundance close to 2 atom % of ¹⁵N and 15 atom % for ¹³C. After three months of culture, the labelled biomass of *P. placenta* was thoroughly rinsed with sterile distilled water (ten washes), freeze-dried and ground. The mycelium powder was directly used as substrate in the *P. involutus* pure cultures for enzymatic tests or incorporated into agar plugs (0.5 g for 100 mL) for the controlled mycorrhization experiments in plastic Magenta boxes. The unlabelled *P. placenta* necromass (control) was produced under the same conditions using unlabelled glucose and NH₄ sulfate. Finally, the mean enrichment in the labelled *P. placenta* mycelium was δ¹³C = 434.34‰ and δ¹⁵N = 5074.52‰; and the natural abundance for unlabelled mycelium was δ¹³C = -17.34‰ and δ¹⁵N = 7.93‰. This mycelium powder was produced under sterile conditions through the whole process.

Proteins and chitin quantification from P. placenta necromass

Soluble proteins were extracted from *P. placenta* necromass. Briefly, 20 mg were mixed with 400 μ L of the extraction buffer [Tris HCl 62.5 mM pH 6.8, SDS 2 % (v/v), Glycerol 10 % and DTT 28mM] with a vortex every 15 min for 1 hour. Samples were centrifuged twice at 14,000 rpm for 15 min (at room temperature). Protein concentration was determined according to RC-DC Protein Assay (RC DC Protein Assay 500-0121, Biorad, Hercules, CA) and expressed as mg of protein for g of dry *P. placenta* necromass. Chitin contents of the *P. placenta* were assayed colorimetrically using methods developed by Chen and Johnson (1983). In short, 8 mg of necromass was hydrolyzed for 4 h at 100°C with 6 M hydrochloric acid. Hydrolysate was then filtrated, evaporated and redissolved in distilled water. Colorimetric reaction was conducted exactly as detailed in Chen and Johnson (1983). Chitin content was expressed in % of dry *P. placenta* necromass, with an adjustment of 9% for loss due to hydrolysis (calculated in this study).

Plant growth conditions and fungal inoculation

First, plastic Magenta boxes were sterilized, hermetically closed and filled with 170 mL of sterilized perlite, an inert mineral substrate poorly enriched in carbon (0.03%) and nitrogen (0.006%). In each box, the perlite substrate was then inoculated with 4 agar plugs colonized by *P. involutus*. During this transfer, 70 mL modified MMN liquid medium (without malt extract) was added to improve the growth of the fungus. In parallel, Scots pine (*Pinus sylvestris*) seeds were surface-sterilized with 50% hydrogen peroxide (H₂O₂) for 30 min, rinsed in distilled water and pre-germinated aseptically on Petri agar plates (agar 10 g/L) at 20°C under a 16-hour photoperiod (Heller et al. 2008). After seven days, the Scots pine seedlings were transferred into Magenta boxes in which the sterile perlite substrate had been previously colonized by *P. involutus*. After 4 months of incubation in growth chamber at 24°C with a 16-h photoperiod providing approximately 275 μ moles/m²s (four lights – 4x58W per shelf), we observed an abundant development of lateral roots on the pines inoculated with *P. involutus* and the formation of the first mycorrhizae (Figure S2). Fungal necromass was then introduced into Magenta boxes under sterile conditions (mycelium powder incorporated into agar plugs). In each box, four patches were positioned on the surface of the perlite layer, providing approximately 30 mg dry organic matter per box. Two treatments were initiated: a “labelled” condition with patches contained labelled ¹³C and ¹⁵N necromass and a control condition with unlabelled patches. In addition, to control a potential ¹³CO₂ flux (carbon mineralization and EM

fungus respiration), a third condition (gas control) was introduced in this experiment (Figure S1). One plastic Magenta box containing a non-mycorrhized seedling, without necromass patch was connected to another Magenta box containing a mycorrhized seedling in presence of labelled necromass (n=3).

All the treatments were maintained for six months at 24°C with a 16-h photoperiod providing approximately 275 $\mu\text{moles}/\text{m}^2\text{s}$ (four lights – 4x58W per shelf), and the sterile conditions were regularly inspected. At the end of the experiment, plants were harvested, and the ectomycorrhizal root tips were sampled under a stereomicroscope, excluding the biological material in direct contact with the labelled patches. Moreover, needles, stems and the perlite colonized with *P. involutus* mycelium were carefully collected.

Extracellular N-acetylglucosaminidase activity of *P. involutus* in the response of *P. placenta* necromass in pure culture

Extracellular exo-chitinolytic activity of *P. involutus* was measured in pure culture in the presence or absence of labelled and unlabelled saprotrophic fungal necromass. For this experiment, *P. involutus* was grown in autoclaved liquid medium comprising: 1 g.l⁻¹ glucose, 0.1 g.l⁻¹ of (NH₄)₂C₄H₄O₆, 1 g.l⁻¹ of KH₂PO₄, 0.5 g.l⁻¹ of MgSO₄·7(H₂O) and 1 mL of microelement solution (see above). This medium was adjusted to pH 5 and autoclaved. *P. placenta* necromass (20 mg of powder) was added to 40 mL of the sterile medium to give a final concentration of 500 mg of necromass per litre of liquid medium. A total of 2.4 mL of liquid medium with unlabelled necromass and liquid medium with labelled necromass was dispensed in a 6-well cell culture plate. From Petri dishes culture (MMN medium), fungal disks (5 mm) of *P. involutus* were cut close to the growth hyphal front and placed in each well (n=6 wells per treatment). The EM fungus was grown in these wells on a layer of autoclaved glass beads immersed in liquid medium complemented with *P. placenta* necromass. After 20 days of growth at 20°C in the dark, the liquid medium of the living *P. involutus* treatments was collected for N-acetylglucosaminidase assay, and the fungal material was sampled separately for isotopic analyses (see below). Moreover, to check potential ¹⁵N and ¹³C labelling contamination resulting from passive adsorption of the labelled material (*P. placenta* powder) on the EM fungus mycelium, in another treatment *P. involutus* mycelium patches were also freeze-dried and introduced in cell culture plate (n=4 wells per treatment). In the same way as for living *P. involutus* mycelia, after 20 days, the dead *P. involutus* disks were collected for isotopic analyses (see below).

N-acetylglucosaminidase activities were measured with enzymatic fluorescence microplate assays adapted from Pritsch et al. (2004). Briefly, 200 μ L of *P. involutus* liquid culture was centrifuged (4500 x g) for 5 min. One hundred microlitres of the supernatant was transferred to a 96-well plate and incubated with 100 μ L acetate buffer (pH=5, 28.82 mL 1 M acetic acid, 273.3 mL 0.3 M sodium acetate and 1 L distilled water) and 50 μ L exo-chitinase substrate, MU-N-acetyl- β -D-glucosaminide, diluted in acetate buffer (pH=5). After 40 min incubation at 23°C, 100 μ L stopping buffer (Tris 2.5 M, pH 10–11) was added with 100 μ L of the incubation solutions. Finally, 100 μ L of this solution was transferred to reading plates. Measurements were performed with the microplate reader Victor3 (Wallac Perkin–Elmer Life Sciences, Villebon-Sur-Yvette, France). The measured fluorescence was expressed in pmol of 4-methylumbelliferone formed by mL of culture medium per min. Since the fungal necromass could conserve residual extracellular enzymatic activities, we also measured the N-acetylglucosaminidase basal activity of the fungal necromass (Brábcová et al., 2016).

Isotopic measurement

Isotopic analysis

The analyses of ^{15}N and ^{13}C isotopes were processed using an online continuous flow CN analyser (Carlo Erba NA1500) coupled with an isotope ratio mass spectrometer (Finnigan delta S). Values were reported in the standard notation ($\delta^{13}\text{C}$ ‰ and $\delta^{15}\text{N}$ ‰) relative to Pee-Dee Belemnite for C, using PEF (IAEA-CH-7) as a standard, and relative to atmospheric N_2 for N, using $(\text{NH}_4)_2\text{SO}_4$ (IAEA-N-1) as a standard. $\delta X = (R_{\text{sample}}/R_{\text{standard}}) - 1) \times 1000$, where R is the molar ratio $^{\text{heavy}}\text{X}/^{\text{light}}\text{X}$ (Zeller et al. 2008).

Incorporation of the brown-rot fungal necromass by EM fungus in pure culture

The living mycelium of *P. involutus* and the freeze-dried mycelium patches used for the N-acetylglucosaminidase assay were collected and placed in a 6-well culture plate with distilled water and agitated during 1 hour to remove any necromass fragments. This washing step was repeated 5 times. Then, dead (patches) and living mycelia were freeze-dried, weighed and used for the isotope analyses. The isotope analyses were conducted on dead and living mycelia of *P. involutus* from the pure culture experiment to measure the direct incorporation of ^{15}N and ^{13}C in the EM fungus from the *P. placenta* necromass.

Estimation of nutrient transfer from necromass to EM plant in monoxenic conditions

In the mycorrhized pine experiment containing labelled ^{13}C and ^{15}N necromass, the isotopic enrichment was measured in the four biological compartments: needles, stems, mycorrhized roots and the perlite colonized by the mycelium of *P. involutus*. The isotopic enrichment was also quantified in the agar patches containing the lignolytic fungal necromass before and after consumption by the ectomycorrhizal fungus to provide the proportion of N and C mobilized during the experiment. Data were collected as atom percentage ^{13}C and ^{15}N and as percentage C and N (relative abundance - $\delta\%$). The excess mass of ^{13}C and ^{15}N was calculated separately using mean biomass of roots, stems and needles. Biomass *P. involutus* mycelium within the perlite could not be measured. Because this value was extremely low in comparison to plant biomass, the proportions of C and N mobilization were estimated by the difference in relative abundance ($\delta\%$) between labelled and unlabelled perlite / mycelium mixture.

Statistics

Statistical analyses were conducted with the R software (R Core Team, 2016). Variables expressed as $\delta^{13}\text{C}$ (‰) and $\delta^{15}\text{N}$ (‰) were analysed using Kruskal-Wallis test by ranks and Wilcoxon signed-rank test. The threshold of significance was fixed for $P < 0.05$. Similarly, enzymatic activities were statistically analysed using Kruskal-Wallis test by ranks.

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TABLE 1. Average values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ for living (n=6) or freeze-dried (n=4) *Paxillus involutus* mycelium in pure-culture experiment in the absence or presence of labelled and unlabelled saprotrophic fungal necromass.

	<i>No necromass</i>		<i>Unlabelled necromass</i>		<i>Labelled necromass</i>	
	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)
EM mycelium	-13.41 (0.5) b ns	7.34 (2.0) c ns	-12.68 (0.2) b ns	8.77 (3.9) b **	123.50 (33.6) a ***	947.66 (91.2) a ***
Freeze-dried EM mycelium	-12.21 (0.1) b	3.28 (0.6) b	-12.38 (0.1) c	2.62 (0.5) c	-3.65 (1.1) a	29.49 (5.7) a

The freeze-dried *P. involutus* mycelium condition allows controlling potential ^{15}N or ^{13}C labelling contamination resulting from passive adsorption from the labelled material. The values in the brackets correspond to the standard deviation. Differences in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures were statistically analysed using Kruskal-Wallis test by ranks (the different letters indicate a significant difference with $P < 0.05$) for necromass type treatments and Wilcoxon signed-rank test ($P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ ***) for *P. involutus* mycelium type treatments.

TABLE 2. Average values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ for monoxenic *P. involutus* / *Pinus* experiment. Values were obtained from all biological compartment studied (mycelium/perlite, EM roots, stem and needles) and from “necromass plugs” in enriched (n=5) and natural abundance (n=5) conditions.

	<i>Unlabelled necromass (P. placenta)</i>		<i>Labelled necromass (P. placenta)</i>	
	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)
Patch (before the experiment)	-17.34 (0.12) b	7.93 (0.27) a	434.34 (19.14) a *	5074.52 (34) a *
Patch (end of the experiment)	-16.79 (0.02) ab	4.31 (1.26) bc	289.21 (46.72) ab ns	3162.41 (579.5) a ns
Perlite + EM mycelium	-12.31 (0.6) a	2.29 (0.3) d	2.12 (7.1) b **	79.37 (39.7) c **
Mycorrhized roots	-22.71 (1.4) c	4.60 (0.8) b	-9.52 (4.3) c ***	341.67 (47.7) b ***
Stem	-24.33 (1.8) d	2.09 (0.4) d	-19.85 (1.6) d **	310.68 (60.4) b **
Needle	-24.26 (1.3) d	3.63 (0.9) c	-17.20 (3.6) d **	311.39 (122.1) b **

The values in the brackets correspond to the standard deviation. Differences in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures were statistically analysed using Kruskal-Wallis test by ranks (the different letters indicate a significant difference with $P < 0.05$) for compartment treatments and Wilcoxon signed-rank test ($P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ ***) for necromass type treatments.

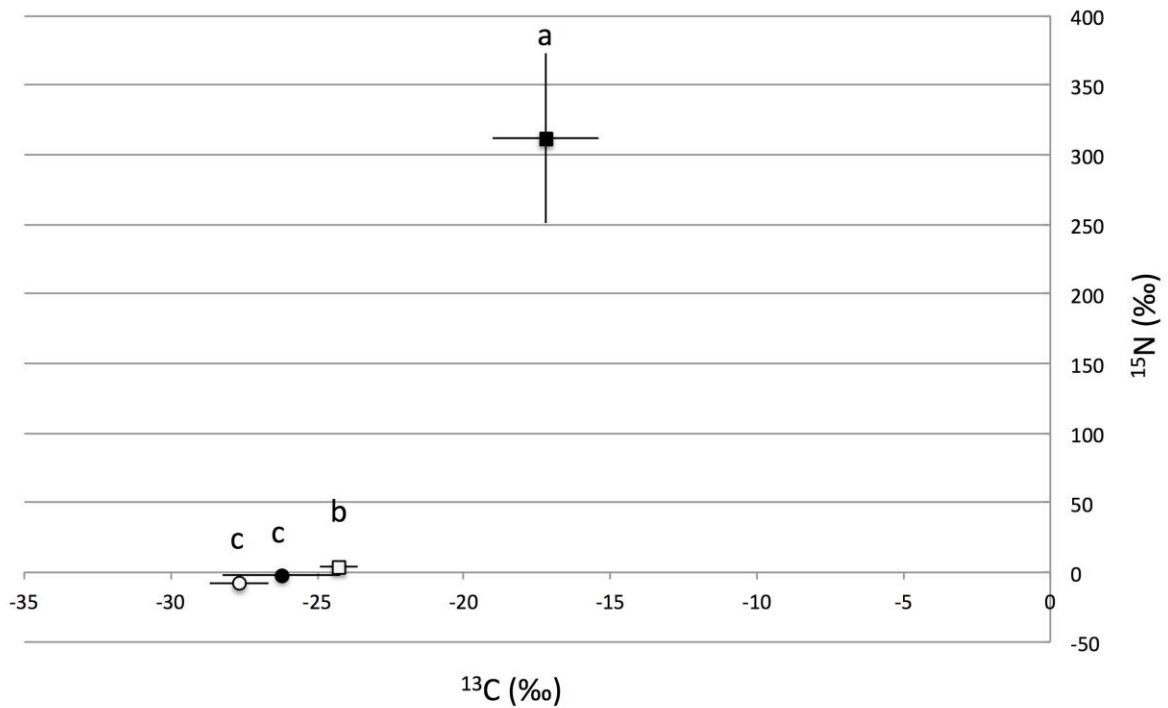


FIGURE 1. Measures of ^{15}N and ^{13}C (expressed as $\delta^{13}\text{C}$ ‰ or $\delta^{15}\text{N}$ ‰) transfers in pine needles, in presence of labelled or unlabelled *P. placenta* necromass. Black square: mycorrhized pines with labelled necromass (n=5); white square: mycorrhized pines with unlabelled necromass (n=5); black circle: control#1 (non-mycorrhized pines with labelled necromass; n=2); white circle: control#2 (non-mycorrhized pines for $^{13}\text{CO}_2$ trapping - experimental device is illustrated in Fig. S1; n=2). See all details in Experimental Procedures section. Differences in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures were statistically analysed using Kruskal-Wallis test by ranks (the different letters indicate a significant difference with $P < 0.05$).

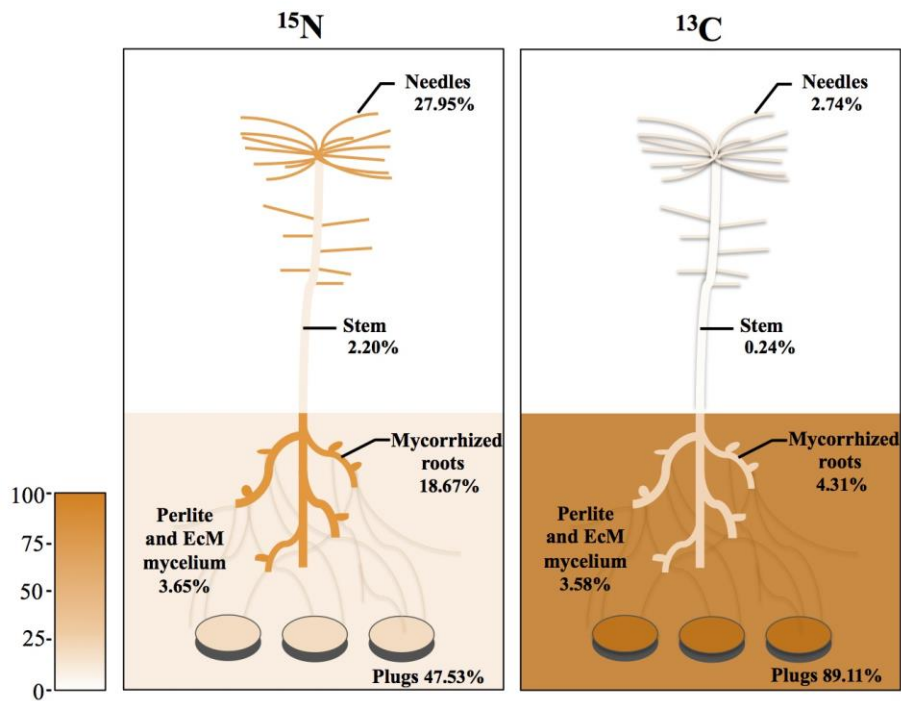


FIGURE 2. Repartition model of the initial ^{15}N (a) and ^{13}C (b) within the different compartments of the tree - EM fungus symbiotic interaction from the inoculum patches of freeze-dried *P. placenta* (n=5). At the end of the experiment, the labelled patches were carefully separated from the other underground compartments (EM roots and perlite - EM mycelium). The compartment “Perlite - EM mycelium” corresponds to the non-plant material present in the plastic Magenta boxes. The “mycorrhized roots” compartment consisted of the entire root system. The allocation percentages were computed as atom percentage ^{13}C and ^{15}N and weighted by biomass of biological material in each compartment. Relative abundance ($\delta\%$) values were used for EM mycelium only, because the mycelium biomass could not be quantified and it was negligible compared to plant biomass.



FIGURE S1. Experimental design to measure the potential EM fungal respiration and $^{13}\text{CO}_2$ transfer in a Magenta box. A. Magenta box with a non-mycorrhizized seedling (Scots pine), without *P. placenta* necromass. B. Magenta box with a mycorrhizized seedling, with *P. placenta* labelled necromass. Boxes were connected with a flexible and hermetic tube.

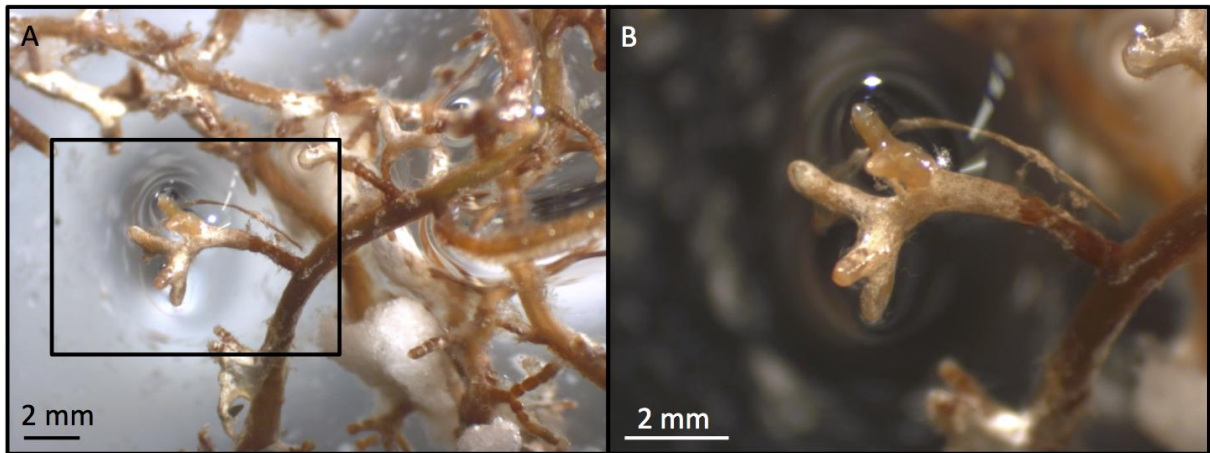


FIGURE S2. A. Image of the mycorrhizal association between *Pinus sylvestris* and *Paxillus involutus* in the Magenta boxes. B. Detail of an ectomycorrhizal root tip.

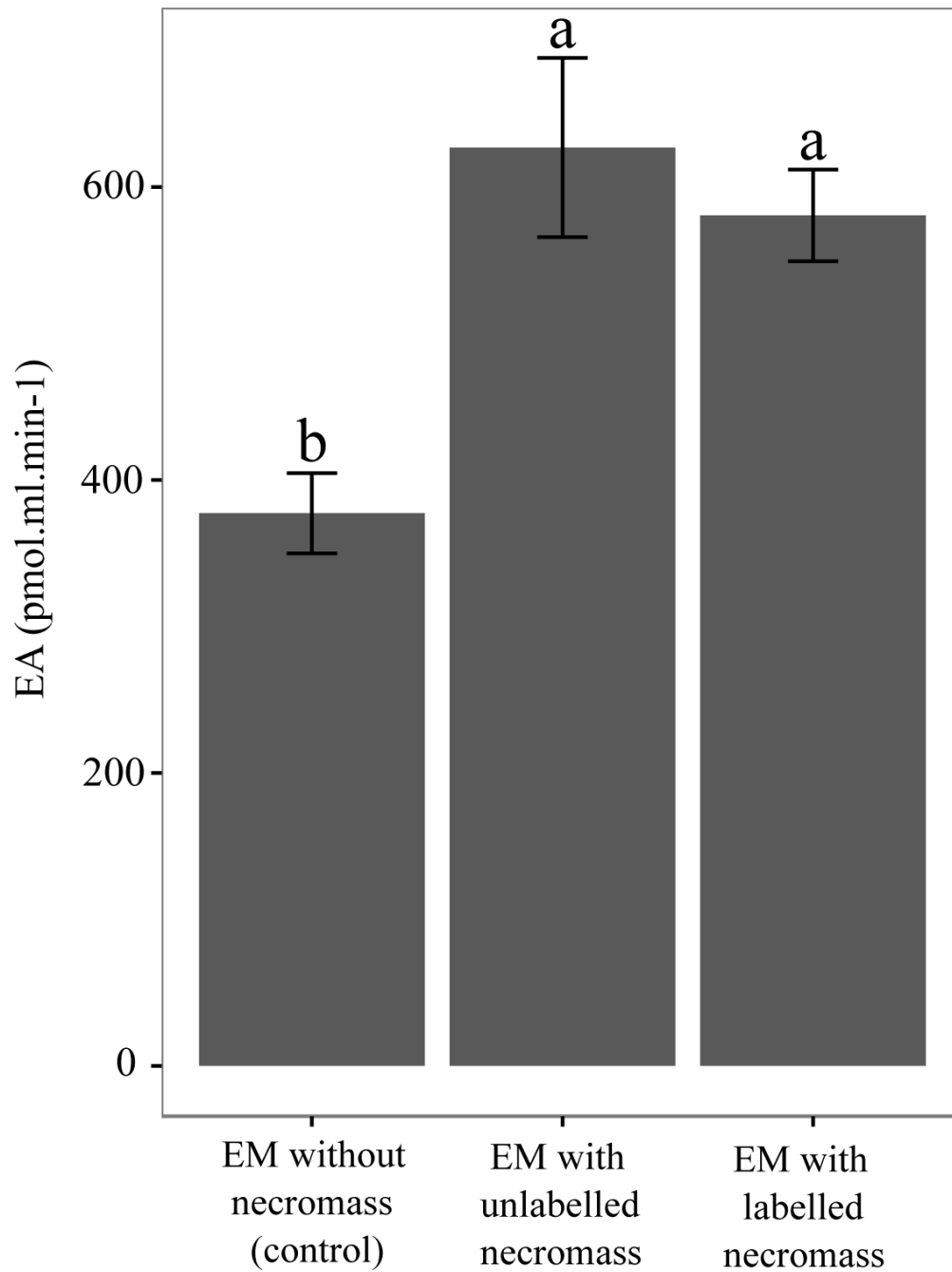


FIGURE S3. Chitinase (N-acetylglucosaminidase) activities of *Paxillus involutus* cultivated in the presence or absence of labelled and unlabelled saprotrophic fungal necromass from *P. placenta* (n=6 in each condition). Differences in N-acetylglucosaminidase activities were statistically analysed using Kruskal-Wallis test by ranks. The different letters indicate a significant difference ($p_{val}<0.05$). The error bars represent the standard deviation.

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CHAPITRE V

N-Acetylglucosaminidase activity, a functional trait of chitin degradation, is regulated differentially within two orders of ectomycorrhizal fungi:

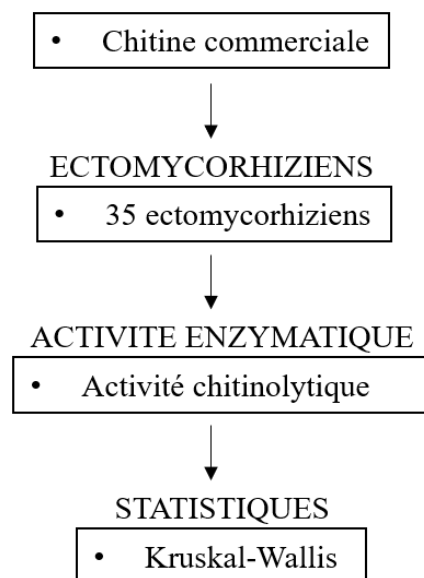
Boletales and Agaricales

Article de recherche publié dans la revue Mycorrhiza, le 13 Avril 2018

INTRODUCTION AU CHAPITRE V

Article de recherche publié dans la revue *Mycorrhiza*, le 13 Avril 2018

Le chapitre V vise à évaluer les capacités chitinolytiques d'une large collection de souches ectomycorhiziennes. Nous avons sélectionné 35 espèces ectomycorhiziennes appartenant majoritairement au phylum des basidiomycota. En culture pure, nous avons comparé les activités enzymatiques de dégradation de la chitine avec et sans chitine ajoutée dans le milieu liquide de croissance des champignons ectomycorhiziens au cours du temps.



N-acetylglucosaminidase activity, a functional trait of chitin degradation, is regulated differentially within two orders of ectomycorrhizal fungi: Boletales and Agaricales

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ABSTRACT

Chitin is one of the most abundant nitrogen-containing polymers in forest soil. Ability of ectomycorrhizal (EM) fungi to utilize chitin may play a key role in the EM symbiosis nutrition and soil carbon cycle. In forest, EM fungi exhibit high diversity, which could be based on function partitioning and trait complementarity. Although it has long been recognized that closely related species share functional characteristics, the phylogenetic conservatism of functional traits within microorganisms remain unclear. Because extracellular N-acetylglucosaminidase activity has been proposed as functional trait of chitin degradation, we screened this activity on thirty-five EM fungi species with or without chitin in the growth medium to i) describe the functional diversity of EM fungi and ii) identify potential links between this functional trait and EM fungal phylogeny. We observed large variations of the extracellular N-acetylglucosaminidase activities among the fungal strains. Furthermore, our results revealed two regulation patterns of extracellular N-acetylglucosaminidase activities. Indeed, these chitinolytic activities were stimulated or repressed in presence of chitin, in comparison to the control treatment. These profiles of extracellular N-acetylglucosaminidase stimulation / repression might be conserved at a high phylogenetic level in the Basidiomycota phylum, as illustrated by the opposite patterns of regulation between Boletales and Agaricales. Finally, the down-regulation of this activity by chitin, for some EM fungal groups, might suggest another chitin degradation pathway.

Keywords: chitin degradation, chitinase, N-acetylglucosaminidase, functional trait, Fungal phylogeny

INTRODUCTION

In temperate and boreal ecosystems, nutrient-poor soils are largely dominated by plant species forming ecto- or ericoid mycorrhizal associations (Clemmensen et al., 2013; Uroz et al., 2016). Nitrogen bioavailability often limits the forest net primary productivity (Rennenberg and Dannenmann, 2015). However, EM fungi are able to exploit the soil organic nitrogenous reserves to supply nitrogen to their host plants (Chalot and Brun, 1998). In these forest soils, a major fraction of the organic nitrogen is embedded into biopolymers. One of these most abundant nitrogen-containing polymers in soil is chitin which contains about 6% of nitrogen (Hodge et al., 1995). Chitin is a linear homopolymer composed of β -1,4-linked subunits of N-acetylglucosamine. Chitin is the second most ubiquitous natural polysaccharide on earth, after cellulose (Dutta et al., 2004). In forest, it is the main component of invertebrate exoskeletons, like arthropods and nematodes, and an essential structural component of the fungal cell walls. Nevertheless, chitin quantification in forest soils remains poorly studied and the contribution of these different organisms to the total pool of soil chitin remains unknown. Several studies reported the presence of chitin in fungal cell walls, and for basidiomycetes, it comprises 16% of the dry weight of the organism (Dahiya et al. 2006). In 1979, Baath and Soderstrom determined that up to 20% of the total soil nitrogen might be incorporated into dead and alive fungal mycelium. As a consequence, the ability of EM fungi to utilize chitin may play an important role for the nutrition either of the fungal symbiont and his host plant. Chitin is very efficiently degraded by certain microorganisms using specific enzymes to break down large polymers of chitin into assimilable molecules (Gaderer et al., 2017a). Firstly, chitinases are involved in the enzymatic degradation of chitin releasing short-chain of chitooligosaccharide with a minimum of two N-acetylglucosamine molecules called chitobiose (Horn et al., 2006). Chitobiose can be converted into N-acetylglucosamine with the action of NAGase (N-acetylglucosaminidase). Finally, N-acetylglucosamine can serve as carbon and/or nitrogen source for fungi (Gaderer et al., 2017a). If Lindahl and Taylor (2004) demonstrated that a wide range of EM Basidiomycota have the genetic potential to produce NAGase, EM fungal root morphotypes expressed this function at variable activity level in field conditions (Buée et al., 2007; Courty et al., 2005, 2010; Hupperts et al., 2017). Furthermore, in temperate and boreal forest ecosystems, EM fungi exhibit high diversity and, as example a single tree can host more than 120 species of EM fungi (Bahram et al., 2011). This huge diversity could be explained by function partitioning and trait complementarity among EM fungal species (Bruns, 1995; Dahlberg, 2001). In vitro experiments showed that EM fungi were able to grow in presence of

chitin as sole nitrogen source, producing variable levels of extracellular chitinase and NAGase activities (Hodge et al., 1995; Leake and Read, 1990). Together, these studies strongly suggest that EM fungi are able to degrade chitin and metabolize nutrients from this biopolymer, but that this functional trait could be differentially distributed among species. However, only a few EM fungal strains were screened for extracellular NAGase activity. In the present study we propose to measure extracellular NAGase activity of thirty-five EM fungi species (more than 20 genera) in presence, or not, of chitin in the growth medium using enzymatic approach based on fluorogenic 4-methylumbelliferyl substrate. Our aims were to i) describe and cluster the different NAGase activity potentials within a representative range of EM fungal genera from Northern Hemisphere Forest, with particular emphasis on two orders (Boletales and Agaricales) and ii) identify potential links between this functional trait and the phylogenetic positioning of EM Basidiomycota.

MATERIALS AND METHODS

Fungal Strain identification

The NAGase activity screening was applied on thirty-five EM fungal strains, composed by 33 basidiomycetes and 2 ascomycetes (Table S.1.), selected from the INRA-IAM unit collection. A large proportion of these strains, isolated from temperate forests of Northern Hemisphere, belong to Boletales and Agaricales orders. Taxonomic identification was confirmed by sequencing of the internal transcribed spacer (ITS) regions, using the fungal specific primer pair ITS1F-ITS4 (Gardes and Bruns, 1993), and Sanger sequences were compared with UNITE database (<https://unite.ut.ee/repository.php>).

Growth conditions

The collection of EM fungal strains were cultivated on sterile cellophane membrane covering solid MNC medium (Yamada and Katsuya, 1995) in darkness at 24 °C. Extracellular NAGase activity of the 35 EM fungal species was measured from liquid medium, in presence or in absence of chitin. For this enzymatic screening, the initial medium was modified to allow carbon and nitrogen deficiencies. The liquid medium was composed with 1 g.l⁻¹ of glucose, 0.1 g.l⁻¹ of (NH₄)₂C₄H₄O₆, 1 g.l⁻¹ of KH₂PO₄, 0.5 g.l⁻¹ of MgSO₄ (H₂O)₇, 1 ml of kanieltra solution, completed or not with 500 mg of practical grade chitin purified from shrimp shell. Media were adjusted to pH 5 and autoclaved. Then, 2.4 ml of liquid medium, with and without chitin, were dispensed in a 6 wells cell culture plate. For the thirty-five EM fungal strains, small mycelium

disks (5mm of diameter, close to the growth hyphal front), were cut from the cellophane membrane and placed in each well (n=3 wells per treatment), then incubated in the dark at 24°C for 32 days. Fungi were grown in wells on a layer of autoclaved glass beads immersed in liquid medium.

Extracellular N-Acetylglucosaminidase activity

Each 8 days, 80 µl of growth culture medium were aseptically collected for the NAGase activity assay. NAGase activity was measured with enzymatic fluorescence tests adapted from (Pritsch et al., 2004). Briefly, 80µl of the growth liquid medium were centrifuged (4500G) for 5 min to remove the chitin powder and the possible fungal mycelium fragments. Then 40 µl of the supernatant were transferred in a 96 wells plate and incubated with 40µl of acetate buffer (pH=5, 28.82 mL of 1M acetic acid, 273.3 mL of 0.3M sodium acetate and 1L of distilled water) and 20 µl of N-Acetylglucosaminidase substrate, MU-N-acetyl-β-D-glucosaminide diluted in acetate buffer (500µM). After 40 min incubation at 23°C, 50µl of this incubation solution were added to 50µl of stopping buffer (Tris 2.5 M, pH 10–11) and this mixture was put in reading plates. Measurements were performed with the microplate reader Victor3 (364 nm excitation, 450 nm emission). The measured fluorescence was expressed in pmol of 4-Methylumbelliferone formed by ml of culture medium by min.

Statistical analysis

Statistical analyses were done with the R software (R Core Team, 2015). Datasets normality was assessed using Shapiro-Wilk test. As datasets were not normally distributed we used non-parametric Wilcoxon test for paired samples. The threshold of significance was fixed for Pval=0.05.

RESULTS AND DISCUSSION

Thirty-five EM fungi were screened for their extracellular NAGase activities with and without chitin in the growth medium. After 8 days of growth, and during all the screening period (32 days), all EM fungal strains showed new mycelium branching, indicating that they were metabolically active. The 35 EM strains used in the present study expressed extracellular NAGase activities highly variable (Table S.2.). If a main part of EM fungi exhibited a relatively low chitinolytic activity, NAGase activity of some EM fungi ranged from 1000 to 6000 pmol.ml⁻¹.min⁻¹ all along the time-course, as illustrated with *Botelus edulis*, *Imleria badia*, *Tricholoma matsutake*, *Tricholoma populinum*, *Amanita muscaria*, *Lanmaoa fragrans* (Table

S.2.). Similar enzymatic variabilities between EM fungi species, or at the intraspecific level, has been previously described for protease activities (Guidot et al. 2005; Nygren et al., 2007) and phenol-oxydase activities (Bending and Read, 1997).

Extracellular NAGase activity was measured in both treatments, with and without chitin (Table S.2.). Extracellular NAGase activity of fungi was not necessarily inducible by chitin, as previously reported by Hodge et al. (1995). In contrast to many other carbohydrate-active enzymes that are solely involved in substrate degradation for nutritional purposes, chitinases have multiple functions in fungal biology as autolysis, nutrition, competition and parasitism (Hartl et al., 2012; Karlsson et al., 2016; Seidl, 2008). The basal extracellular NAGase activities measured in the growth media without chitin could be explained by some of these diverse roles, in particular the mycelium recycling. For these reasons, we only considered enzymatic activity stimulation when NAGase activity was higher in the chitin treatment than in the control. Interestingly, we also observed that for 15 EM fungi, extracellular NAGase activity was lower in the chitin treatment than in the control treatment (Table S.1.). Clustering analysis, based on extracellular NAGase activity fold change (chitin over control), revealed two distinct patterns of responses to the substrate for EM fungi, suggesting that chitin could play as a stimulator or a repressor for this activity (Fig 1.a.). Regarding these two patterns, extracellular NAGase activities of *Tuber borchii*, *Cortinarius glaucopus*, *Cenococcum geophilum*, *Thelephora terrestris* and of *Laccaria*, *Hebeloma*, *Tricholoma* and *Amanita* genera were mainly stimulated all along the time-course experiment in presence of chitin. Stimulation of NAGase activity in presence of chitin, in comparison with the control, could indicate degradation mechanisms of this polymer by these EM fungi. For this group of fungi, we can thus hypothesize that extracellular NAGase activity was stimulated to hydrolyse chitin polymers into metabolisable N-acetylglucosamine units.

Conversely, extracellular NAGase activity of *Scleroderma citrinum*, *Gyrodon lividus*, *I badia*, *Piloderma sp*, *Suillus luteus*, *Pisolithus tinctorius*, and strains belonging to the genus of *Leccinum*, *Paxillus*, *Rhizopogon* and *Thelephoraceae* family, were mainly repressed when the medium was complemented with chitin (Fig 1.a.). Even if the repression of NAGase activity in presence of chitin remains difficult to explain, we can propose different hypotheses. First, in a nutritional context, it appears uncommon to observe a decrease of the degrading enzyme activity in presence of the substrate. Utilization of other degradation pathway(s) could explain repressed profiles measured in our study. Moreover, Hodge et al. (1995) reported the lack of correlation between NAGase activity and growth rate for pathogenic and EM fungi in presence

of chitin, suggesting that fungi could incorporate directly non-completely hydrolyzed chitin oligomers into their cell wall. This mechanism would bypass the catabolism pathway and might be more energy efficient. If some EM fungi produced endochitinase activity, as it was demonstrated by (Hodge et al., 1995), we could propose that fungi which repressed NAGase activity, avoid consequently formation of N-acetylglucosamine to conserve potential assimilable chitin oligomer. Nevertheless, this hypothesis is currently not supported by published results. Furthermore, another degradation pathway could de-acetylated chitin into chitosan, and then degraded by chitosanases enzymes. Fungal chitosanases mainly belong to the GH75 family and act on the reducing end of the chitosan molecule and thereby produce glucosamine oligosaccharide (Cheng et al., 2006). Finally, another chitin degradation pathway by fungi has been more recently demonstrated. It is based on the action of lytic polysaccharide monooxygenases (LPMOs) that cause random chain breaks into cellulose, hemicellulose and chitin (Beeson et al., 2012; Hemsworth et al., 2013). Indeed, Gaderer et al. (2017b) showed that *Neurospora crassa*, a filamentous fungus, presented a better growth in presence of chitin but was partially repressed with the addition of N-acetylglucosamine. As genes coding NAGase activity were not induced in presence of chitin they suggested that *N crassa* potentially used LPMOs to degrade chitin. They proved that degradation of chitin by fungi can be disconnected from the chitinase and NAGase pathways. In consequence, NAGase activity cannot be considered as a unique and universal functional marker of chitin-degrading capabilities in all fungi. Genomic data could validate, or not, the hypothesis of these alternative pathways within this group of Basidiomycetes.

In a non-nutritional hypothesis it is also important to report that N-acetylglucosamine can occasionally inhibit the growth of fungi (Naseem et al., 2011). As reported by Gaderer et al. (2017b), spore germination and mycelium growth of *N crassa* were affected by N-acetylglucosamine supply, even at low concentration. We also can propose that some groups of fungi have negative feedback regulation. This inhibition would occur when the final product of the reaction (N-acetylglucosamine) interferes with the NAGase. This inhibition could be done as a regulatory mechanism to limit potential toxicity of the final product beyond a critical concentration in the growth medium.

Interestingly, extracellular NAGase activity stimulation and repression patterns appeared to be well conserved in Agaricales and Boletales fungal orders, respectively. Indeed, the mean fold change (chitin over control) was 2.3 for the Agaricales EM fungi and -1.8 for the Boletales EM

fungi (p -value < 0.001) (Fig 1.B.). Conservatism, within Agaricales and Boletales fungi, of these two pathways of NAGase regulation in presence of chitin has never been described previously. Interestingly, Mucha et al. (2006) observed a very low extracellular NAGase activity for *Suillus* strains which belong to Boletales order in presence of colloidal chitin in comparison with *Laccaria* and *Amanita* strains, belonging to Agaricales order. In our study, some EM fungi, belonging to the Thelephorales order, showed the two different profiles of regulation. In one hand, NAGase activity of the two *Thelephoraceae* strains were repressed in presence of chitin, and in the other hand NAGase activity of *Thelephora terrestris* was mainly stimulated in presence of this substrate. Consequently, if the two patterns of NAGase regulations can be connected to both taxonomic groups Agaricales and Boletales fungi, it is difficult to interpret the results for the other orders due to small number of fungal strains used. The temporal measures of NAGase activities within Agaricales and Boletales orders revealed two different time periods of NAGase activity regulation (Fig 2.). The extracellular NAGase activity was not significant at 8 days (p -value > 0.05) for the Boletales fungi, but significant for the other time points (p -value < 0.05) (Fig 2.A). For the Agaricales fungi, the stimulation of extracellular NAGase activity was immediately significant, until 24 days (p -value < 0.05), but not at 32 days (p -value > 0.05) (Fig 2.B). For both orders (Boletales and Agaricales) and the two treatments (with and without chitin) we measured an increase of the extracellular NAGase activity during the two first weeks, then the curves reached a plateau after three weeks (Fig 2.). A carbon and/or a nitrogen starvation could explain these curve profiles, particularly because C and N concentrations in our liquid medium were relatively limited.

Extracellular enzymatic activities of fungi could be used as functional traits (Buée et al. 2007; Cullings and Courty, 2009). Moreover, Talbot et al. (2015) reported that enzymatic profiles could predict the fungal functional group between EM and saprotrophic fungi. In the same way, Mathieu et al. (2013) separated ecological subgroups, as brown, white and soft rot fungi, using enzymatic profiling. If some functional traits of AM fungi appear to be similar among species relatively close within broad phylogenetic groupings, particularly at the family level (Powell et al. 2009; Chagnon et al. 2013, Treseder and Lennon, 2015), little is known for EM fungi (Koide et al., 2014). There appears to be a need for better identification of traits and understanding of mycorrhizal fungal response traits, including ericoid fungi (Koide et al. 2014). Indeed, coupling these functional approaches with recent genomic data, traits could participate to an efficient categorisation of fungi according to lifestyles and highlight ecological questioning like the divide between ericoid and saprotrophs (Selosse et al. 2018). In the present study, extracellular

NAGase activity of EM fungi is strain dependent but its regulation seemed to be conserved at a high phylogenetic level between Agaricales and Boletales EM fungi. Such phylogenetic conservatism indicates that phylogeny would be a potential proxy for predicting chitin turnover in forest soils and the relative performance of EM fungi in the field. Nevertheless, as already discussed, NAGase activity is not the only way to metabolize chitin by fungi. In consequence, this potential functional indicator could be associated to other traits, or functional molecular markers, involved in microbial necromass degradation, as illustrated for lignocellulolytic functions (Bödeker et al., 2012; Barbi et al. 2016)

To conclude, extracellular NAGase activity revealed important variabilities between the different fungal strains studied in the present screening. Interestingly, chitin could act as a stimulator or repressor of extracellular NAGase activity for EM fungi, suggesting a sensitive negative feedback of this activity for Boletales. We proposed that Agaricales and Boletales EM fungi presented two distinct NAGase profiles in response to chitin availability, corresponding to potential phylogenetic conservatism of the regulation of this function for these two fungal orders. The use of a wider fungal strain collection would allow broadening this hypothesis of phylogenetic conservatism of certain functional traits in fungi. Finally, as reported for plant cell wall degrading enzymes (Kohler et al., 2015), a comparative genomic analysis of genes that code for chitin degradation is still needed to better understand the regulation of chitinolytic activities among EM fungi and the potential different pathways involved in the degradation of this polymer.

CONFLICT OF INTEREST

The authors declare no conflict of interest

ACKNOWLEDGMENTS

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FIGURE CAPTIONS:

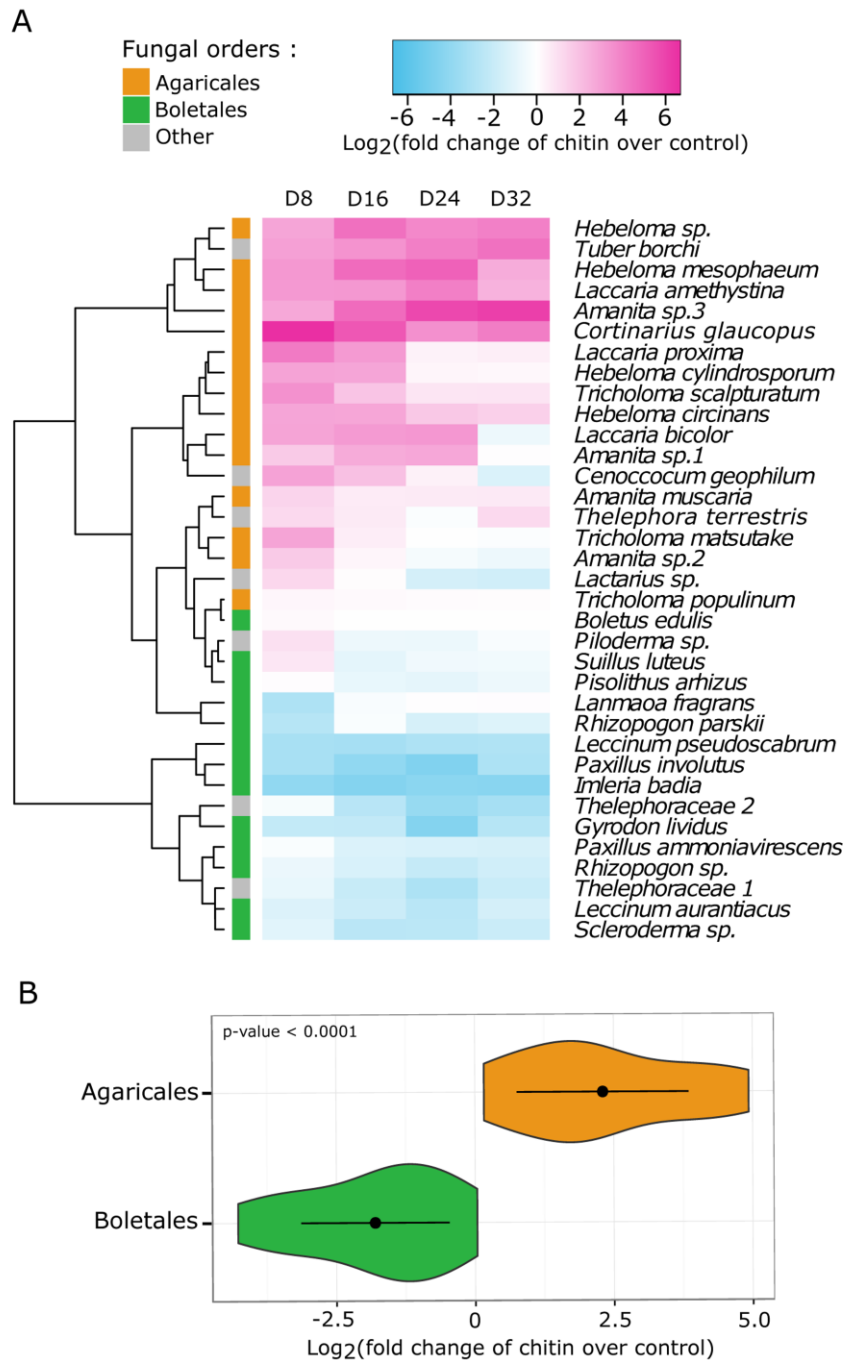


FIGURE.1. (a) Heatmap showing EM fungi extracellular NAGase activity fold changes (chitin treatment over control treatment) at four growth time points clustered by euclidean distance. (b) Violin plot of mean NAGase activity fold changes (chitin treatment over control treatment) between EM fungi belonging to Agaricales (n=15species) and Boletales (n=13 species) orders. Growth time points were averaged for each EM fungal strain. Extracellular NAGase activity fold changes were compared using Wilcoxon test for paired samples.

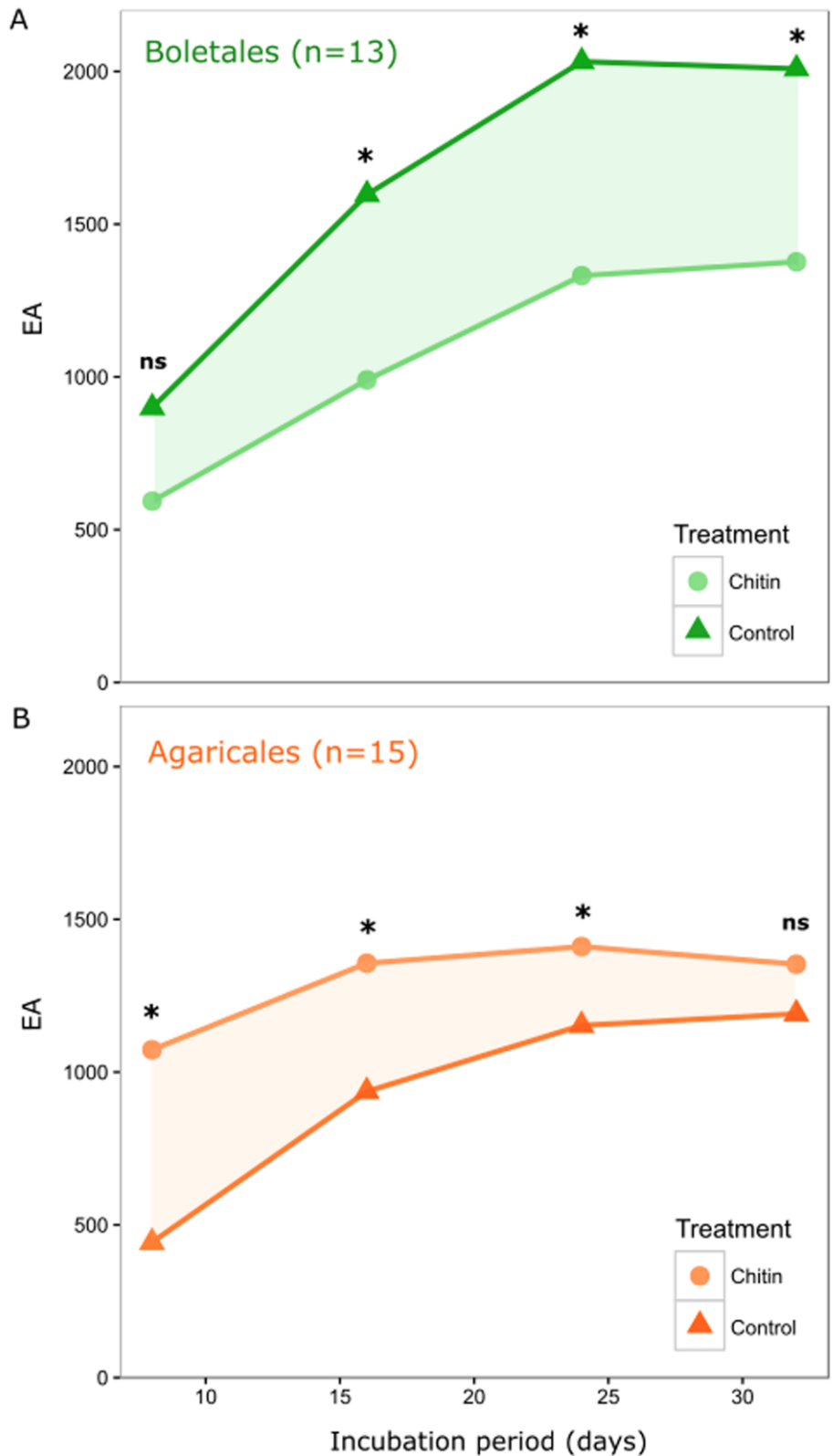


FIGURE. 2. Extracellular NAGase activity (pmol.ml⁻¹.min⁻¹) in function of growth time (in days) for EM fungi belonging to Agaricales (n=15species) and Boletales (n=13 species) orders with and without chitin in the growth medium. Extracellular NAGase activity fold changes were compared using Wilcoxon test for paired samples.

SUPPLEMENTARY MATERIAL:

TABLE S.1. Ectomycorrhizal fungi used in the present study.

Fungal strain	(UNITE best match)	Order	Strain code
<i>Amanita muscaria</i>	AB080795 (99/100)	Agaricales	Koide 1998
<i>Amanita sp.1</i>	FN610865 (674/675)	Agaricales	MB2
<i>Amanita sp. 2</i>	AY194966 (640/644)	Agaricales	42
<i>Amanita sp. 3</i>	HM146791 (668/671)	Agaricales	MB1
<i>Boletus edulis</i>	KP031593 (734/736)	Boletales	Hub 8'
<i>Cenococcum geophilum</i>	JX630518 (775/784)	Mytilinidiales	1.58
<i>Cortinarius glaucopus</i>	KJ421061 (668/668)	Agaricales	AT 2004 276
<i>Gyrodon lividus</i>	DQ534568 (739/739)	Boletales	BX
<i>Hebeloma circinans</i>	JF908041 (630/633)	Agaricales	BR10
<i>Hebeloma cylindrosporum</i>	KX687197 (674/678)	Agaricales	H7
<i>Hebeloma sp.</i>	UDB008778 (630/631)	Agaricales	BRU
<i>Hebeloma mesophaeum</i>	JQ724062 (675/676)	Agaricales	GRAM
<i>Laccaria amethystina</i>	JX504150 (665/668)	Agaricales	LaAM-08-1
<i>Laccaria bicolor</i>	KC881087 (666/668)	Agaricales	S238N
<i>Laccaria proxima</i>	JX907813 (666/668)	Agaricales	S106
<i>Lactarius sp.</i>	KT334798 (719/720)	Russulales	S23C
<i>Lanmaoa fragrans</i>	UDB000946 (722/723)	Boletales	Bf 92 - 1
<i>Leccinum aurantiacum</i>	UDB015695 (431/433)	Boletales	QBC
<i>Leccinum pseudoscabrum</i>	UDB015533 (409/411)	Boletales	CNRF1
<i>Paxillus ammoniavirescens</i>	KX610700 (724/730)	Boletales	pou 09.2
<i>Paxillus involutus</i>	AJ438984 (742/744)	Boletales	01
<i>Piloderma sp.</i>	HG937066 (605/610)	Atheliales	F1598
<i>Pisolithus arhizus</i>	KJ809558 (641/643)	Boletales	MARX 270
<i>Rhizopogon sp.</i>	JQ975972 (680/682)	Boletales	KTP
<i>Rhizopogon parksii</i>	KC306756 (688/690)	Boletales	SMITH
<i>Scleroderma sp.</i>	KT334789 (657/658)	Boletales	Foug A
<i>Suillus luteus</i>	KX230641 (677/679)	Boletales	UH-Slu-Lm8-n1
<i>Thelephora terrestris</i>	DQ822828 (648/650)	Russulales	813
<i>Thelephoraceae 1</i>	FJ455018 (641/655)	Russulales	Lentang
<i>Thelephoraceae 2</i>	JQ975992 (652/654)	Russulales	AT
<i>Tricholoma populinum</i>	UDB023599 (633/638)	Agaricales	BLAE
<i>Tricholoma scalpturatum</i>	EU160589 (666/666)	Agaricales	AUL
<i>Tricholome matsutake</i>	AB368513 (668/670)	Agaricales	945
<i>Tuber borchii</i>	KT165350 (540/541)	Pezizales	17B0
<i>Imleria badia</i>	KY693727 (383/383)	Boletales	84.06

TABLE S.2. Extracellular N-acetylglucosaminidase activity (pmol.ml⁻¹.min⁻¹) of ectomycorrhizal fungi with and without chitin in the growth medium in function of growth time (Values are means (\pm SE; n = 3)).

	Day 8		Day 16		Day 24		Day 32	
	Control	Chitin	Control	Chitin	Control	Chitin	Control	Chitin
<i>Amanita muscaria</i>	1433 +/- 182	3726 +/- 90	2840 +/- 159	4425 +/- 142	3021 +/- 182	4762 +/- 62	2616 +/- 113	4171 +/- 43
<i>Amanita sp.1</i>	11 +/- 2	35 +/- 4	9 +/- 1	57 +/- 7	15 +/- 3	105 +/- 11	123 +/- 37	126 +/- 18
<i>Amanita sp.2</i>	353 +/- 135	1142 +/- 67	1767 +/- 586	2184 +/- 172	1565 +/- 245	1231 +/- 438	1982 +/- 339	1287 +/- 206
<i>Amanita sp.3</i>	7 +/- 1	52 +/- 14	15 +/- 2	411 +/- 68	9 +/- 1	539 +/- 97	9 +/- 1	641 +/- 114
<i>Boletus edulis</i>	5696 +/- 46	6424 +/- 156	6450 +/- 66	6422 +/- 36	6305 +/- 46	6247 +/- 24	6403 +/- 33	6383 +/- 37
<i>Cenococcum geophilum</i>	10 +/- 1	77 +/- 8	10 +/- 1	40 +/- 6	52 +/- 9	71 +/- 17	354 +/- 41	139 +/- 42
<i>Cortinarius glaucopus</i>	1 +/- 1	130 +/- 7	13 +/- 4	553 +/- 87	34 +/- 4	401 +/- 131	23 +/- 8	420 +/- 113
<i>Gyrodon lividus</i>	37 +/- 5	8 +/- 1	63 +/- 7	13 +/- 1	270 +/- 2	12 +/- 1	246 +/- 1	39 +/- 5
<i>Hebeloma circinans</i>	7 +/- 1	54 +/- 11	19 +/- 8	141 +/- 40	23 +/- 2	80 +/- 28	35 +/- 6	97 +/- 32
<i>Hebeloma cylindrosporum</i>	3 +/- 1	26 +/- 14	6 +/- 2	50 +/- 43	12 +/- 2	15 +/- 13	87 +/- 40	104 +/- 87
<i>Hebeloma sp.</i>	5 +/- 2	40 +/- 4	17 +/- 10	409 +/- 153	22 +/- 5	308 +/- 111	23 +/- 7	382 +/- 128
<i>Hebeloma mesophaeum</i>	2 +/- 2	28 +/- 4	2 +/- 1	55 +/- 8	1 +/- 1	25 +/- 7	1 +/- 1	11 +/- 3
<i>Laccaria amethystina</i>	9 +/- 1	91 +/- 3	8 +/- 1	82 +/- 4	6 +/- 1	115 +/- 3	20 +/- 7	111 +/- 9
<i>Laccaria bicolor</i>	19 +/- 9	145 +/- 29	15 +/- 1	144 +/- 30	22 +/- 3	224 +/- 25	314 +/- 39	202 +/- 25
<i>Laccaria proxima</i>	26 +/- 2	500 +/- 187	274 +/- 60	448 +/- 117	629 +/- 91	834 +/- 138	698 +/- 98	1004 +/- 209
<i>Lactarius sp.</i>	476 +/- 315	1158 +/- 89	374 +/- 168	395 +/- 58	763 +/- 242	260 +/- 46	842 +/- 260	261 +/- 74
<i>Lanmaoa fragrans</i>	343 +/- 63	43 +/- 13	4071 +/- 380	3465 +/- 392	5671 +/- 48	5838 +/- 56	5707 +/- 64	6011 +/- 64
<i>Leccinum aurantiacum</i>	298 +/- 12	123 +/- 29	638 +/- 4	173 +/- 25	629 +/- 6	107 +/- 7	578 +/- 35	194 +/- 12
<i>Leccinum pseudoscabrum</i>	304 +/- 8	33 +/- 9	230 +/- 10	24 +/- 4	346 +/- 45	45 +/- 8	327 +/- 13	44 +/- 10
<i>Paxillus ammoniavirescens</i>	40 +/- 6	34 +/- 18	324 +/- 26	129 +/- 35	659 +/- 75	247 +/- 60	716 +/- 68	251 +/- 64

<i>Paxillus involutus</i>	325 +/- 35	35 +/- 4	360 +/- 58	22 +/- 3	570 +/- 103	23 +/- 8	589 +/- 105	74 +/- 11
<i>Piloderma sp.</i>	525 +/- 182	1025 +/- 96	709 +/- 4	457 +/- 104	1131 +/- 119	704 +/- 190	875 +/- 48	744 +/- 115
<i>Pisolithus arhizus</i>	127 +/- 42	132 +/- 32	532 +/- 40	300 +/- 40	890 +/- 61	459 +/- 159	871 +/- 129	555 +/- 95
<i>Rhizopogon sp.</i>	27 +/- 12	16 +/- 4	66 +/- 34	25 +/- 6	164 +/- 146	38 +/- 12	261 +/- 193	79 +/- 41
<i>Rhizopogon parksii</i>	3 +/- 1	1 +/- 1	23 +/- 2	20 +/- 1	17 +/- 3	6 +/- 2	76 +/- 22	32 +/- 14
<i>Scleroderma sp.</i>	539 +/- 204	255 +/- 73	565 +/- 206	98 +/- 40	895 +/- 267	162 +/- 62	902 +/- 251	236 +/- 79
<i>Suillus luteus</i>	215 +/- 51	370 +/- 17	3998 +/- 185	2023 +/- 313	5561 +/- 68	3887 +/- 366	5324 +/- 90	3788 +/- 322
<i>Thelephora terrestris</i>	11 +/- 3	26 +/- 3	74 +/- 12	117 +/- 47	100 +/- 20	88 +/- 45	123 +/- 12	284 +/- 12
<i>Thelephoraceae 1</i>	188 +/- 36	106 +/- 30	179 +/- 21	43 +/- 8	278 +/- 34	34 +/- 70	241 +/- 28	62 +/- 19
<i>Thelephoraceae 2</i>	110 +/- 63	90 +/- 16	94 +/- 53	15 +/- 1	194 +/- 83	13 +/- 1	245 +/- 64	26 +/- 1
<i>Tricholoma matsutake</i>	684 +/- 154	5122 +/- 577	3463 +/- 598	5025 +/- 623	6073 +/- 259	6170 +/- 306	6043 +/- 118	5433 +/- 107
<i>Tricholoma scalpturatum</i>	4076 +/- 280	4982 +/- 119	5567 +/- 257	6245 +/- 77	5805 +/- 121	6262 +/- 39	5811 +/- 13	6175 +/- 123
<i>Tricholome populinum</i>	1 +/- 1	17 +/- 8	29 +/- 16	109 +/- 34	47 +/- 9	83 +/- 27	69 +/- 11	124 +/- 8
<i>Tuber borchii</i>	28 +/- 8	230 +/- 23	108 +/- 45	1162 +/- 210	67 +/- 27	206	77 +/- 31	1761 +/- 281
<i>Imleria badia</i>	3732 +/- 39	232 +/- 105	3437 +/- 81	152 +/- 65	4432 +/- 87	231 +/- 39	4102 +/- 43	204 +/- 62

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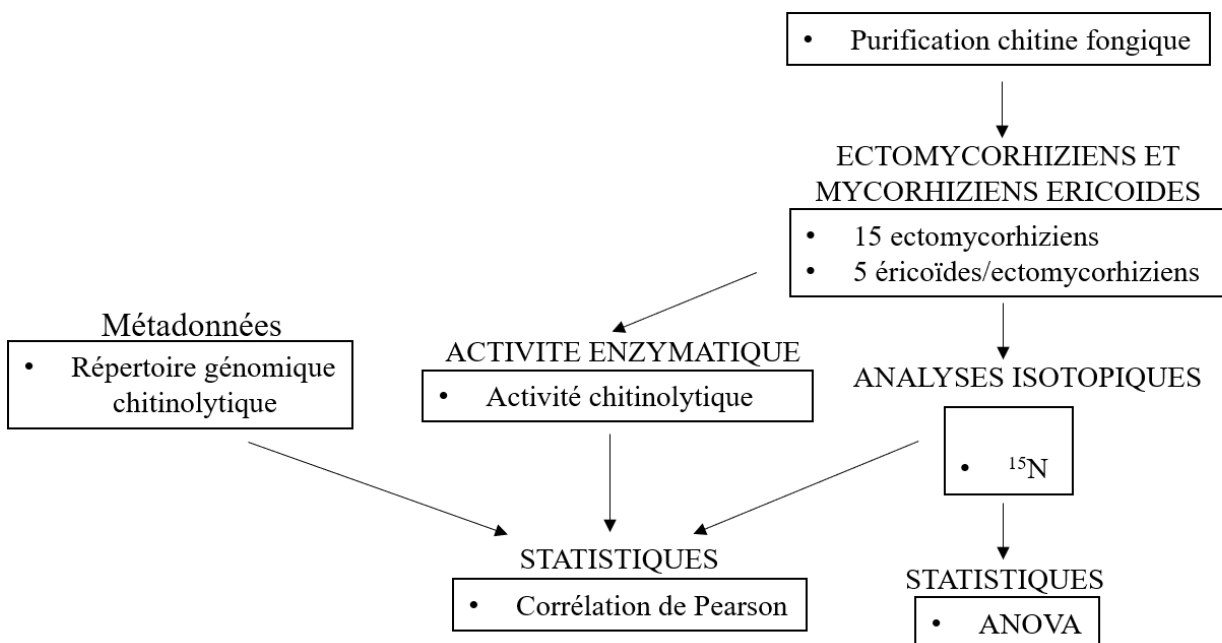
CHAPITRE VI

Organic nitrogen mobilisation from fungal chitin was revealed by genomics and physiological evidences in a wide range of ectomycorrhizal fungi

Article de recherche en préparation pour la revue Mycorrhiza

INTRODUCTION AU CHAPITRE VI

Le chapitre VI concerne l'étude des capacités de dégradation de la chitine de souches ectomycorhiziennes. Par des approches isotopiques et enzymatiques, nous avons mesuré les capacités de mobilisation d'azote provenant de chitine fongique purifiée pour 15 souches d'espèces ectomycorhiziennes dont les génomes ont été séquencés. Les capacités chitinolytiques de ces 15 souches ectomycorhiziennes ont été comparées à 5 espèces éricoïdes. De plus, le potentiel génomique de dégradation de la chitine et ces données fonctionnelles ont ensuite été mis en lien.



Organic nitrogen mobilisation from fungal chitin was revealed by genomics and physiological evidences in a wide range of ectomycorrhizal fungi

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Abstract

- Ectomycorrhizal (EcM) fungal symbionts play crucial role in nitrogen (N) nutrition of their host trees. Ability of EcM fungi to mobilise N from organic sources such as protein was evaluated but data remain scarce for chitin, another abundant N-containing polymer in forest soil.
- We compared the genomic repertoires involved in chitin degradation of fifteen EcM fungi with five ericoid (ErM) mycorrhizal fungi and notably *Rhizoscyphus ericae*, well described as efficient chitin degrader. We measured the ability of these EcM and ErM fungal species to mobilise organic N from fungal chitin using enzymatic and ¹⁵N isotopic labelling approaches. In parallel, we used genomics resources to linked gene repertoires of these mycorrhizal fungi, potentially involved in chitin degradation, with the functional screening.

- Some EcM fungi presented an important number of gene families coding chitinases (AA11, GH18 and GH20), notably *Boletus edulis* and *Xerocomus badius*. Furthermore, *Boletus edulis*, *Xerocomus badius*, *Suillus luteus* and *Hebeloma cylindrosporum* were able to efficiently mobilise organic ¹⁵N from chitin. We demonstrated positive correlation between genomics and functional potentials for chitin degradation within the EcM fungi guild.
- We demonstrated a high capacity of some EcM fungi for fungal chitin degradation, suggesting that soil organic N mining by EcM fungal community would result both from proteolysis and chitin degradation. We proposed chitinase activity as a discriminant functional trait of EcM for their capacity to absorb various forms of soil N for tree nutrition.

Keywords: Ectomycorrhizal fungi, Ericoid mycorrhizal fungi, Chitin, Chitinase, Organic nitrogen mobilisation.

INTRODUCTION

Forest primary productivity is mainly limited by soil fertility, in particular nitrogen (N) availability (Näsholm et al., 2009; Rennenberg and Dannenmann, 2015). In these N limited ecosystems, ectomycorrhizal (EcM) fungi play key role in the N nutrition of their host trees acting like a functional extension of the roots (Mayor et al., 2015, Nehls and Plassard, 2018). If inorganic N mobilisation and transfer to associated trees by EcM fungi is well described (Finlay et al., 1992; Marschner and Dell, 1994), the knowledge of the mobilization processes of the many forms of organic N by EcM fungi remains still fragmented (Pellitier and Zak, 2018). However, recent studies shown a growing interest in this “organic nutrient use hypothesis” (Dickie et al., 2014) and its vast ecological implications. Indeed, organic N mining by EcM fungi could directly contribute to tree N nutrition and therefore to forest primary productivity. Moreover, competition between EcM and saprotroph fungi for organic N could slowdown organic matter decomposition rates contributing to increase soil C stocks (Orwin et al., 2011; Averill et al., 2014; Fernandez and Kennedy, 2016). Consequently, identify EcM fungi implicated in organic N mining as well as the N-containing molecules they degrade represents a major challenge to understanding primary productivity and geochemical cycles in forest (Lindahl and Tunlid, 2015).

Organic N mining by EcM fungi was until now exclusively attributed to protein degradation (Op De Beeck et al., 2018). Proteolytic activities of EcM fungi were extensively studied and a classification between “protein degrader” and “non-protein degrader” EcM fungi has been proposed (Abuzinadah et al., 1986; Bending and Read, 1996; Chalot and Brun, 1998; Nygren et al., 2007; Shah et al., 2013). Additionally to proteolytic activities, it has been demonstrated that some EcM fungi can attack organic matter using enzymatic (i.e. manganese peroxidase) and non-enzymatic mechanisms (i.e. Fenton reaction) to increase the availability of its associated N (Bödeker et al., 2014; Rineau et al., 2016, Shah et al., 2016; Op De Beeck et al., 2018; Nicolás et al., 2018). Furthermore, degradation by EcM fungi of particular N-containing polymers such as chitin, remains little explored despite the fact that chitin represents an important N pool in soil (Sowden et al., 1978; Stevenson 1982; Gonzalez-Prieto and Carballas, 1991; Kögel-Knabner, 2002).

This major component of arthropods and fungal cell wall (Kumar, 2000) consists in a linear homopolymer of N-acetylglucosamine β -1,4-linked subunits. Chitin is decomposed by microorganisms using chitinases and β -N-acetylglucosaminidases (NAGases) liberating N-acetylglucosamine which is most available form for the fungi (Gaderer et al., 2017). In addition, lytic polysaccharide monooxygenases (LPMOs) represent another group of enzymes implicated in chitin degradation by causing random chain breaks (Beeson et al., 2012; Hemsworth et al., 2014). Akroume et al. (2018) depicted that *Paxillus involutus*, an EcM fungus, was able to efficiently mobilise and transfer N to host seedlings from fungal necromass. Moreover, it is reported that ectomycorrhizae and EcM fungi mycelium in pure culture secreted chitinases and NAGases (Rineau and Garbaye, 2009; Jones et al., 2012; Walker et al., 2016; Hupperts et al., 2017; Nicholson and Jones, 2017; Maillard et al., 2018). As Chitinases and NAGases are potentially involved in different fungal biological processes such as cell wall remodelling (Langner and Göhre, 2016; Langner et al., 2015; Shin et al., 2009) and fungal-fungal competition (Lindahl and Finlay, 2006), it remains difficult to conclude about the exclusive nutritional purpose of this enzyme secretion by EcM fungi.

In the present study, we explored the genomic resources and measured the functional capacities of EcM and ericoid mycorrhizal (ErM) fungi to degrade fungal chitin and mobilise the released N. We compared EcM fungi to ErM fungi because these fungi were recently described as efficient chitin degraders based on their genomics resources (Martino et al., 2018). Moreover, the ErM fungus *Rhizoscyphus ericae* is the only described plant-associated fungus able to degrade chitin and transfer organic N to associated plant (Kerley and Read, 1995; Leake and

Read, 1990). We firstly used comparative genomics approach based on data matching the number of carbohydrates-active enzymes (CAZymes) potentially implicated in chitin degradation (Cantarel et al., 2009, Floudas et al., 2012; Knapp et al., 2018; Martino et al., 2018; Kohler et al., 2015; Nagy et al., 2016). In this aim, we analysed the genomic repertoires for chitin degradation of twenty fungi species belonging to EcM and ErM fungal guilds. Secondly we used isotopic approach based on ¹⁵N mobilisation from isotopic labelled chitin to assess the ability of these twenty fungal species to mobilise organic N from sterile fungal chitin in pure culture. Due to the important variation in secreted NAGase activity observed recently in a large set of EcM fungal species (Maillard et al., 2018), we hypothesised that organic N mobilisation from chitin by the EcM fungi would be highly variable and species-dependent. Finally, we tried to bridge the genomic potentials and functional activities for chitin degradation of screened fungi based on correlation approach. Indeed, as comparative genomics studies postulate that genes repertoire size could mirror fungal functional potential (Kohler et al., 2015; Martino et al., 2018), we tested hypothesis that functional and genomic potentials for chitin degradation would be positively correlated.

MATERIALS AND METHODS

Comparative genomics approach

Genomic repertoire for chitin degradation

We used twenty EcM and ErM or EcM/ErM fungi strains (Table 1) whose genome was sequenced by the Mycorrhizal Genomics Initiative Consortium (<http://genome.jgi-psf.org/programs/fungi/index.jsf>). These fungal strains were chosen because they cover a broad phylogenetic diversity within Basidiomycota and Ascomycota phyla. Based upon Langner and Göhre (2016) review, we selected two glycoside hydrolases (GH) families (GH18 and GH20) and one auxiliary activity (AA) family (AA11) as proxy of genomic repertoire for chitin degradation. Additionally, we determined the putatively secreted genomic repertoire for chitin degradation for these twenty fungal strains using the secretome prediction pipeline defined in Pellegrin et al (2015) for the three Cazymes families (GH18, GH20 and AA11).

Phylogeny construction

We constructed a phylogeny of the twenty selected fungal strains. We identified orthologous genes from 20 fungi using FastOrtho with the parameters set to 50% identity, 50% coverage,

inflation 3.0 (Wattam et al., 2014). Protein sequences used for the process were genome-wide protein assemblies from JGI fungal portal MycoCosm. We identified clusters with single copy genes. We aligned each cluster with MAFFT 7.221 (Katoh et al., 2013), we eliminated ambiguous regions (containing gaps and poorly aligned) and we concatenated single-gene alignments with Gblocks 0.91b (Castresana 2000). We constructed phylogenetic tree with RAxML 7.7.2 (Stamatakis 2006) using the standard algorithm, the PROTGAMEWAG model of sequence evolution and 1,000 bootstrap replicates. This phylogenetic tree was used as a support to illustrate the quantitative data obtained from both functional approaches and genomic repertoire for chitin degradation (Figure 1).

Functional approach

Fungal ¹⁵N chitin extraction from Postia placenta

We extracted chitin from *Postia placenta* necromass as defined by (Ospina Álvarez et al., 2014). *P. placenta* was grown in agitated liquid medium composed with 100% of (¹⁵NH₄)₂SO₄. After two months of growth, we collected *P. placenta* mycelium using a sterile sieve. We crushed the *P. placenta* biomass in a sterile shaker during 10 min. Then, we washed the biomass with distilled water for five times to remove growing medium and water-soluble compounds. Then we applied two successive deproteinization treatments with sodium hydroxide solution (NaOH) at 4M with a ratio of 1: 30 (w/v) for 2 hours at 100°C. Then, we washed *P. placenta* biomass with distilled water until rich pH 7 in solution. Finally, we dried and weighted extracted fungal chitin. We quantified the purity of the extracted fungal chitin as described in Chen and Johnson (1983). Briefly, 8 mg of chitin were hydrolysed for 4h at 100°C with 6M hydrochloric acid. We filtrated, evaporated and dissolved the fungal chitin hydrolysate in distilled water. We conducted the colorimetric reaction as detailed in Chen and Johnson (1983). Chitin purity was expressed as % of glucosamine per chitin dry mass, with an adjustment of 9% for loss due to hydrolysis (calculated in this study). We assessed in the same way commercial chitin from shrimp shell (Sigma-Aldrich, France). The purity of fungal chitin we extracted was very similar to that of commercial chitin from shrimp shell (Sigma-Aldrich, France), with respectively 43% and 47% in glucosamine equivalent. These results are close to other chitin purity assessments (Ospina Álvarez et al., 2014). We have also extracted soluble proteins from fungal extracted chitin and commercial chitin. In this aim, 20 mg of chitin were mixed with 400µL of the extraction buffer [Tris HCl 62.5 mM pH 6.8, SDS 2 % (v/v), Glycerol 10 % and DTT 28mM] with a vortex every 15 min for 1 hour. Samples were centrifuged twice at 14.000 rpm for 15

min. Finally we determined protein concentration according to RC-DC Protein Assay (Biorad RC DC Protein Assay 500-0121). Results indicate a very low level of soluble protein content for isolated fungal chitin (0.1% dry matter), which is slightly higher than commercial shrimp shell chitin (0.04% dry matter). We proceeded with the analyses of ^{15}N isotopes of extracted fungal chitin using an online continuous flow CN analyser (Carlo Erba NA1500) coupled with an isotope ratio mass spectrometer (Finnigan delta S). Isotopic analyses indicated that fungal extracted chitin was ^{15}N labelled at 83%.

Experimental design

We cultivated fungal strains on sterile cellophane membrane covering solid MNC medium (Yamada and Katsuya, 1995) in darkness at 24 °C. To quantify the ^{15}N mobilization from chitin by the 20 fungal strains, we set up an experiment in dual solid / liquid pure culture medium. Indeed, to avoid that labelled chitin fragment adhered accidentally to the growing mycelium, ^{15}N labeled chitin was entrapped in agar in the bottom of six wells plates. In this way, we dispensed in each well of a six well plate 1 ml of an autoclaved medium composed of 500 mg.l⁻¹ labelled chitin and 8 g.l⁻¹ of agar. To dispense an equal quantity of chitin in each well, medium was kept under agitation during the addition of this agar layer in the wells. For the control treatment, we added 1ml of 8 g.l⁻¹ agar medium without chitin in the wells. After agar polymerization, we added in each well 2.4 ml of a carbon and nitrogen deficiencies liquid medium composed with 1 g.l⁻¹ of glucose, 0.1 g.l⁻¹ of $(\text{NH}_4)_2\text{C}_4\text{H}_4\text{O}_6$, 1 g.l⁻¹ of KH_2PO_4 , 0.5 g.l⁻¹ of $\text{MgSO}_4 (\text{H}_2\text{O})_7$, 0.025 g.l⁻¹ of NaCl , 0.050 g.l⁻¹ of CaCl_2 and 1 ml of kanieltra solution. We adjusted the pH of the medium to pH 5 prior autoclaving. We added autoclaved glass beads in liquid medium within each well to support to growth of the fungi. For the 20 fungal strains, we cut small mycelium disks (5mm of diameter) close to the growth hyphal front from the cellophane membrane and placed one disk in each well (n = 6 wells per treatment per fungal strain). The plates were then incubated in the dark at 24 °C for 28 days. After 28 days of incubation, we collected mycelium of the twenty fungal strains growing in upper liquid layer of both treatments (with and without chitin). The mycelium was dried on a paper towel and immediately stored in liquid nitrogen. Then, all the samples were freeze-dried for isotopic analyses. Each mycelium sample (n=6 per treatment) was weighted and completely used for these analyses. The biomass measured during this step was used to compare the final biomass of the twenty fungal strains grown with and without chitin. The analyses of ^{15}N isotopes were proceed using an online continuous flow CN analyser (Carlo Erba NA1500) coupled with an isotope ratio mass spectrometer (Finnigan delta S). Values concerning ^{15}N incorporation from

chitin by the fungal strains were expressed of ^{15}N atom % excess ($\%^{15}\text{N}_{\text{chitin treatment}} - \%^{15}\text{N}_{\text{control treatment}}$).

Secreted NAGase activity assessment

Just after the harvest of the mycelium, we collected 200 μl of growth culture medium for the NAGase activity assay. We measured secreted NAGase activity with enzymatic fluorescence test as described in Maillard et al. (2018). Briefly, we centrifuged 200 μl of the growth liquid medium (4,500G) for 5 min to remove residual fungal fragments. Then, we transferred 100 μl of the supernatant in a 96-well plate and we incubated this culture supernatant with 100 μl of acetate buffer (pH = 5, 28.82 mL of 1 M acetic acid, 273.3 mL of 0.3 M sodium acetate and 1 L of distilled water) and 50 μl of N-acetylglucosaminidase substrate (Sigma Aldrich Chemicals, Lyon, France) as described in Pritsch et al. (2004). After 40 min of incubation at 23 °C, 100 μl of this incubation solution was added to 100 μl of stopping buffer (Tris 2.5 M, pH 10–11) and this mixture was put in reading plates. Measurements were performed with the microplate reader Victor 3 (364 nm excitation, 450 nm emission). We expressed the measured fluorescence in pmol of 4-methylumbelliferone formed by milliliter of culture medium by minute (EA).

Statistical analysis and data visualisation

Statistical analyses and data visualisation were performed using R software (R Core Team 2016) with ggplot2, ggtree, and egg packages (Auguie 2017, Yu et al., 2017, Wickam 2009). The Shapiro–Wilk test was used to test normality of the data and we compared the functional and genomic chitin degradation data using Student's t-Tests and Pearson's correlations. We consider P-value as statistically significant when the value was less than 0.05 ($p < 0.05$)

RESULTS

Chitin degradation genomic potential

We compared the genomic repertoires involved for chitin degradation of twenty EcM and ErM fungal species (Figure 1). All the fungal species studied presented genes belonging to GH18 and GH20 Cazymes families. Only Ascomycota fungi have AA11 Cazymes family in their genomes. We observed important size variation of genomic repertoires for chitin degradation ranging from 8 genes for *Pisolithus tinctorius* to 33 for *Odiendron maius*. *O. maius*, *Acephala*

macrosclerotiorum, *Meliniomyces variabilis*, *Meliniomyces bicolor*, *Boletus edulis*, *Xerocomus badius* and *Paxillus involutus* presented large genomic repertoires for chitin degradation with a number of total genes superior to 20. Putatively secreted genomic repertoires involved for chitin degradation followed this same trend. Main part of genes coding chitin degradation was predicted secreted.

Chitin degradation functional potential

After 28 days of growth, only *Oidiodendron maius* biomass increased significantly (e.g. 28%) in the presence of chitin compared to treatment without chitin in the growth medium (Figure 1). Secreted NAGase activity appeared to be extremely variable ranging from 0 to 6582 pmol of 4-methylumbelliferone formed per millilitre of culture medium per minute in presence of chitin. Fungi showing important NAGase activity were *X. badius*, *B. edulis*, *Suillus luteus*, *Amanita muscuria*, *Tricholoma matsutake* and *O. maius*. We measured significant increase of secreted NAGase activity in presence of chitin for *Lactarius quietus*, *Piloderma croceum*, *S. luteus*, *O. maius*, *R. ericae*, *M. variabilis* and *M. bicolor*. Conversely, significant decrease of NAGase activity in presence of chitin was observed for two Boletaceae: *X.badius* and *P. involutus*. The mobilisation of N from chitin, which is expressed in ^{15}N atom % excess, broke down into two categories: high or low. Indeed, main part of the fungi screened (13/20) shown very low ability of ^{15}N mobilization from chitin with ^{15}N atom % excess below to 0.01. The other fungi seven strains, *O.maius*, *M.bicolor*, *R.ericcae*, *B.edulis*, *X.badius*, *S.luteus* and *Hebeloma cylindrosporum*, mobilized relatively important ^{15}N from chitin that ranged from 0.03 to 0.14 ^{15}N atom % excess.

Relationships between genomic and functional analyses for chitin degradation

We used Pearson's correlation to test links between the occurrence of three chitinase families (AA11, GH18 and GH20) and functional potentials for chitin degradation for all studied fungal species in our study and for EcM fungi only (Figure 2). We found significant positive correlation ($P=0.022$, $r=0.51$) between genomic repertoires for chitin degradation and NAGase activity in presence of chitin for the twenty fungal species. Similarly, we identified significant and positive correlation ($P=0.024$, $r=0.50$) between putatively secreted genomic repertoires for chitin degradation and NAGase activity for all these studied strains. Interestingly, correlation approaches conducted only on the EcM strains revealed significant and positive correlation

between i) putatively secreted chitinase families and secreted NAGase activity in presence of chitin ($P=0.011$, $r=0.63$), ii) this putatively secreted genomic repertoires for chitin degradation and ^{15}N mobilization ($P=0.011$, $r=0.63$) and iii) NAGase activity in presence of chitin and ^{15}N mobilization ($P=0.0096$, $r=0.64$).

DISCUSSION

EcM fungi genomes contain rich repertoire of genes coding for chitin degradation enzymes

The first study targeting genes encoding putative EcM fungi chitinases revealed by polymerase chain reaction detection the presence of NAGase genes in 18 EcM fungi belonging to twelve different genera (Lindahl and Taylor, 2004). Our data confirmed these results and showed that some EcM fungi possessed an important number of genes encoding chitinase similarly to ErM fungi described as facultative saprotroph symbiotic fungi (Martino et al., 2018). As suggested in our hypotheses, this gene repertoire is quantitatively variable and species-dependent, as already reported for plant cell wall degrading genes (Kohler et al., 2015), lipases (Martino et al., 2018), proteases (Martino et al., 2018) and small secreted proteins (Pellegrin et al., 2015).

Some EcM fungi are efficient chitin degraders

In agreement with previous genomic studies and functional experiments (Kerley and Read, 1995; Leake and Read, 1990; Kerley and Read, 1995; Martino et al., 2018), the ErM fungi *R. ericae*, *O. maius* and *M. bicolor* mobilised efficiently N from fungal chitin. Similarly to these chitin degrader fungi, we demonstrated that *B. edulis* and *X. badius*, two EcM fungi belonging to Boletaceae family, mobilise also efficiently N from chitin. In this way, Rasmussen *et al.* (2018) showed that Boletaceae ectomycorrhizae root tip presented the highest level of NAGase enzyme activity by comparison to other EcM fungal families in a mixed upland forest. *H. cylindrosporum*, another EcM fungi we determined as efficient chitin degrader, produced high amount of chitinases belonging to GH18 family in its culture filtrate (Doré et al., 2015). In the present study, 27% (4/15) of EcM fungi were able to efficiently mine N from fungal chitin. Talbot et al., (2010) estimated that 87% of EcM and ErM fungi studied were able to degrade protein. Chitin degradation appeared to be a functional trait less widespread than protein degradation within EcM fungi. *S. luteus*, *B. edulis* and *X. badius*, which mobilized efficiently ^{15}N from chitin in our study, are phylogenetically relatively close and have a brown rot fungus as ancestor (Kohler et al., 2015). However, other close phylogenetic fungi to these three fungi

we screened such as *P. involutus* and *S. citrinum* did not mobilize N efficiently from chitin. Moreover, *H. cylindrosporium*, which belong to Hymenogastraceae family and would be a descendant of a white rot fungus (Kohler et al., 2015), degraded also chitin efficiently. These results indicated that the chitin degradation ability is presumably genus or species dependent rather than being conserved at higher level of phylogeny as we previously hypothesised. Nevertheless, larger screening would be needed to definitively exclude this hypothesis. Although some EcM fungi are regarded to be efficient organic matter degraders, as for example *P. involutus*, *C. glaucopus*, *T. matsutake* and *P. croceum*, (Bödeker et al., 2014; Heinonsalo et al., 2015; Op De Beeck et al., 2018, Vaario et al., 2018), they were not able to efficiently mobilise N from chitin in our experimental conditions. Because plant litter and the microbial biomass are composed of complex mixtures of organic components (Kögel-Knabner, 2002), the diversity of soil fungi species, including EcM fungi community, could support functional diversity adapted to the variability of polymers and N-containing molecules constituting the organic matter of the soils. Indeed, our physiological results about chitin degradation by EcM fungi could be relied with ecological observations. EcM fungi are one of the main fungal guild in mineral forest soil which is potentially rich in fungal necromass containing chitin (Bahram et al., 2015; Clemmensen et al., 2015). Recent results showed that EcM fungi could rapidly colonize fungal necromass (Brabcová et al., 2016; Fernandez and Kennedy, 2018), which also contains important quantity of chitin (Whipps, 1987). Moreover, EcM fungi exclusion experiment using trench plots in boreal forest revealed a decrease of soil chitobiohydrolase and N-acetylglucosaminidase activities by comparison to control plots (Sterkenburg et al., 2018). Together, these results could indicate a direct role of certain ectomycorrhizal fungi in chitin degradation in forest soils. It should be noted that the observations made in the current study were done in a pure culture system and are probably not a complete mirror of what is occur in forest ecosystem. EcM fungi chitinolytic potential is surely influences by the association of several parameters, like host plant, the season, the climate or the balance between organic and mineral N source in soil (Buée et al. 2007; Rineau et al., 2012). In natural condition, chitin never occurs pure, generally forming complexes with different proteins (Stankiewicz et al., 1998). Because both categories of polymers are potentially degraded by EcM fungi, we could expect concomitant protease and chitinase activities targeting these N-containing organic complexes.

Genomic background and functional chitin degradation measures are positively correlated for EcM fungi

We demonstrated a significant positive correlation between the repertoire of genes involved in chitin degradation within EcM fungal guild and their chitin degradation activities measured through isotopic and enzymatic approaches. This first demonstration of the direct link between genomic background and functional trait quantification related to fungal organic matter degradation confirms our original hypothesis. Furthermore, predicted secreted genomic repertoire for chitin degradation of EcM fungi appears to be better predictor than total genomic repertoire (e.g. secreted and non-secreted proteins) for their degradation potential of this biopolymer. Nevertheless, some EcM fungi displayed high secreted NAGase activities in growth medium (e.g. *T. matsutake*), and other, like *A. muscaria*, shown low chitin degradation capacities. We may attribute these differences to the fact that some secreted enzymes would be essentially EcM fungal cell wall-linked and not secreted in the liquid medium. In this case, the enzymes would not be able to interact with chitin that was trapped in solid medium lower layer. Indeed, it is admitted that some NAGase enzymes, which are described as genomic bottleneck for chitin degradation, are linked to the fungal cell wall (López-Mondéjar et al., 2009). Consecutively, some fungi could not complete their chitin degradation pathway if they mainly possessed cell wall-linked chitinases or cell wall-linked NAGases. We assume that our experimental set-up minimizes the false-positive errors and maximize the false-negative results. Moreover, we observed poor correlation between genomic background and functional measures from the total set of fungi we screened, due to ErM fungi addition that blurred this correlation. Indeed, *A. macrocleriotiorum* and *M. variabilis* which presents an elevated chitinase repertoire, were not able to efficiently mobilize N from chitin in our experiment. In addition, as depicted by Gaderer et al. (2017), chitinases are not only involved for degradation of extracellular chitin, but also in other fungal physiological processes, such as cell wall remodeling during fungal growth or cell wall degradation during autolysis and apoptosis. Consequently, some chitinase families could be jointly proxies of chitin degradation, competition or remodeling potentials. In this case, we could assume that a part of the chitinase gene repertoires of these ErM and ErM/EcM fungi is involved in other functions, like competition as proposed by Leake and Read (1990) and blur our correlation approach. Finally, transcriptomic approaches are now necessary to identify fungal chitinase genes directly implicated in chitin degradation for a nutritional purpose to improve our attempt to bridge functional and genomic chitin degradation potentials.

CONCLUSION

Our data highlight the fact that some EcM fungi present high number in genes potentially implicated in chitin degradation. We demonstrate that some EcM fungal species are able to efficiently mine organic N from fungal chitin in pure culture. Our results therefore provide the first direct evidence about the link between genomic background and functional activities involved for chitin degradation within EcM fungi. We propose that this validated functional trait of EcM fungal symbionts will contribute to increase our understanding of tree N nutrition and soil carbon cycle in forest ecosystems.

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Conflict of interest

We state that there is no conflict of interest.

Author contributions

F.M. and M.B. designed the experiment. F.M., S.M., E.M., C.H., N.A., I.Z., N.B., D.G., F.M.M. and M.B. performed the experiment or collected, analyzed and interpreted the data. F.M. wrote the first draft of the manuscript. All the authors contributed to the final version of the manuscript.

TABLE

TABLE 1. Fungal strains used in this study.

Fungal strain	ID	Lifestyle	Mycocosm link
<i>Amanita muscaria</i>	A.mus	Ectomycorrhizal	https://genome.jgi.doe.gov/Amamu1/Amamu1.home.html
<i>Boletus edulis</i>	B.edu	Ectomycorrhizal	https://genome.jgi.doe.gov/Boled1/Boled1.home.html
<i>Cenococum geophilum</i>	C.geo	Ectomycorrhizal	https://genome.jgi.doe.gov/Cenge3/Cenge3.home.html
<i>Cortinarius glaucopus</i>	C.gla	Ectomycorrhizal	https://genome.jgi.doe.gov/Corgl3/Corgl3.home.html
<i>Gyrodon lividus</i>	G.liv	Ectomycorrhizal	https://genome.jgi.doe.gov/Gyrli1/Gyrli1.home.html
<i>Hebeloma cylindrosporium</i>	H.cyl	Ectomycorrhizal	https://genome.jgi.doe.gov/Hebcy2/Hebcy2.home.html
<i>Laccaria bicolor</i>	L.bic	Ectomycorrhizal	https://genome.jgi.doe.gov/Lacbi2/Lacbi2.home.html
<i>Lactarius quietus</i>	L.qui	Ectomycorrhizal	https://genome.jgi.doe.gov/Lacqui1/Lacqui1.home.html
<i>Paxillus involutus</i>	P.inv	Ectomycorrhizal	https://genome.jgi.doe.gov/Paxin1/Paxin1.home.html
<i>Piloderma croceum</i>	P.cro	Ectomycorrhizal	https://genome.jgi.doe.gov/Pilcr1/Pilcr1.home.html
<i>Pisolithus tinctorius</i>	P.tin	Ectomycorrhizal	https://genome.jgi.doe.gov/Pisti1/Pisti1.home.html
<i>Scleroderma citrinum</i>	S.cit	Ectomycorrhizal	https://genome.jgi.doe.gov/Sclci1/Sclci1.home.html
<i>Suillus luteus</i>	S.lut	Ectomycorrhizal	https://genome.jgi.doe.gov/Suilu4/Suilu4.home.html
<i>Tricholoma matsutake</i>	T.mat	Ectomycorrhizal	https://genome.jgi.doe.gov/Trima3/Trima3.home.html
<i>Xerocomus badius</i>	X.bad	Ectomycorrhizal	https://genome.jgi.doe.gov/Xerba1/Xerba1.home.html
<i>Meliniomyces bicolor</i>	M.bic	Ectomycorrhizal/Ericoid	https://genome.jgi.doe.gov/Melbi2/Melbi2.home.html
<i>Meliniomyces variabilis</i>	M.var	Ericoid mycorrhizal	https://genome.jgi.doe.gov/Melva1/Melva1.home.html
<i>Oidiodendron maius</i>	O.mai	Ericoid mycorrhizal	https://genome.jgi.doe.gov/Oidma1/Oidma1.home.html
<i>Rhizoscyphus ericae</i>	R.eri	Ericoid mycorrhizal	https://genome.jgi.doe.gov/Rhier1/Rhier1.home.html
<i>Acephala macrosclerotiorum</i>	A.mac	Ectomycorrhizal/Ericoid	https://genome.jgi.doe.gov/Acema1/Acema1.home.html

FIGURE CAPTIONS

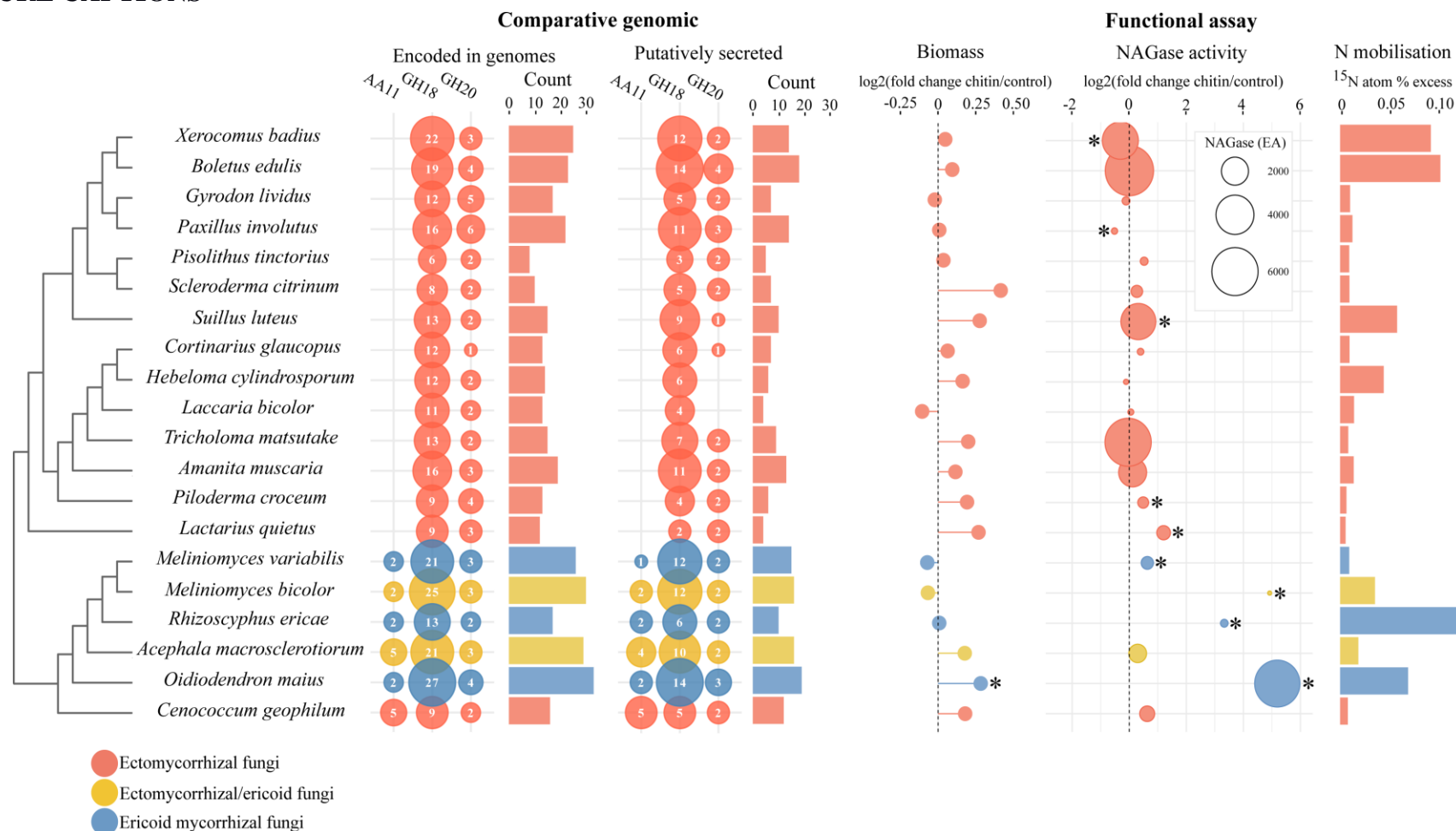


FIGURE 1. Genomic repertoires for chitin degradation (number of genes belonging to GH18, GH20 and AA11 families), predicted secreted genomic repertoires for chitin degradation (number of predicted secreted genes belonging to GH18, GH20 and AA11 families), effect of chitin on biomass and NAGase activity (fold change chitin over control), NAGase activity in presence of chitin, ^{15}N mobilisation from chitin (^{15}N atom % excess) for twenty fungal species screened colored in function of their functional guild (red=ectomycorrhizal fungi, blue=ericoid mycorrhizal fungi, yellow= ericoid and ectomycorrhizal fungi).

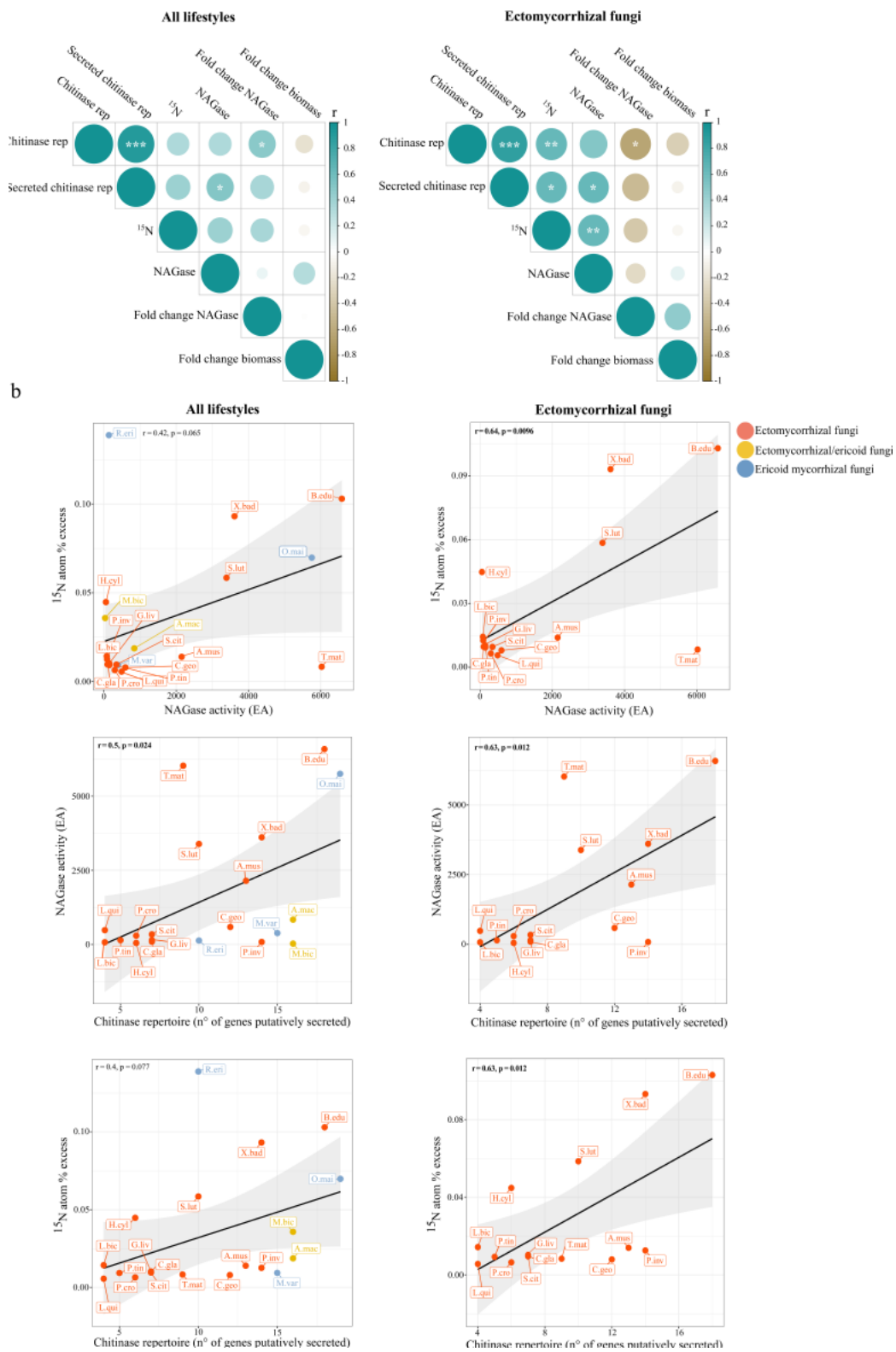


FIGURE 2. a. Pearson correlations between the genomic and functional potentials for chitin degradation for total (n=20) and EcM fungal species (n=15) screened. Colour of the circle depends on the direction of the correlation (green=positive and brown=negative). Size of the circle is proportional to the r value. Stars in the circle indicate significant correlations (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$). Abbreviations: Chitinase rep, genomic repertoires for chitin degradation; Secreted chitinase rep, predicted secreted genomic repertoires for chitin degradation; ¹⁵N, ¹⁵N mobilisation from chitin; NAGase, NAGase activity in presence of chitin; Fold change NAGase, effect of chitin on NAGase activity; Fold change biomass, effect of chitin on biomass. b. Representation of correlations between predicted secreted genomic repertoires for chitin degradation, ¹⁵N mobilisation from chitin and NAGase activity in presence of chitin for total (n=20) and EcM fungal species (n=15) screened.

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DISCUSSION GENERALE ET PERSPECTIVES

DISCUSSION GENERALE ET PERSPECTIVES

1. Les effets de l'exportation artificielle de matière organique sur la diversité taxonomique et fonctionnelle microbienne en forêt tempérée.

L'utilisation des pratiques sylvicoles intensives a généralement débuté en amont de la mise en place de sites expérimentaux de l'étude des impacts de ces pratiques sur l'écosystème forestier (Achat et al., 2015). Ainsi les résultats obtenus sur les effets des pratiques sylvicoles intensives sont simultanés ou postérieurs aux effets mesurés sur les forêts exploitées intensivement. La vocation d'anticipation et d'indication des effets potentiellement négatifs des sites expérimentaux d'études des pratiques sylvicoles est donc limitée. Par conséquent, l'avantage de l'exportation artificielle de matière organique est qu'elle permet d'accélérer nettement le phénomène d'épuisement des écosystèmes forestiers en matière organique et d'en observer les effets sur des forêts en période d'accroissement maximum. En effet, la majorité des études portent sur l'effet des pratiques sylvicoles intensives sur des forêts relativement jeunes et après une rotation de récolte intensive seulement (Hartmann et al., 2012 ; Achat et al., 2015 ; Wilhelm et al., 2017). Les effets du retrait artificiel de matière organique que nous avons mesuré sont résumés dans la figure 4. Les effets négatifs du retrait de matière organique sur les fonctions microbiennes telluriques semblent en cohérence avec les résultats de la méta-analyse de Achat et al. (2015). Les approches enzymatiques sont fréquemment utilisées afin d'étudier le fonctionnement microbien des sols. En complément de ses approches le laboratoire de Biogéochimie des Ecosystèmes Forestiers (BEF) mesure les vitesses de minéralisation de carbone et de l'azote des sols du réseau MOS. Les différences de vitesse minéralisation du carbone et de l'azote des sols entre placettes de références et placettes de retrait de matière organique ne sont pas significatives (résultats non montrés). Ainsi les approches enzymatiques semblent présentées un niveau de sensibilité à la perturbation plus important que les approches de mesure de vitesse de minéralisation. Afin d'évaluer de manière plus précise les effets de l'exportation intensive de matière organique sur les fonctionnements microbiens des sols nous avons conduit un approche méta-transcriptomique. Les résultats méta-transcriptomiques des sols témoins et manipulés sont actuellement en cours d'analyse et devraient nous permettre de mettre en évidence des fonctions bactériennes et fongiques associées à la réponse au retrait de matière organique.

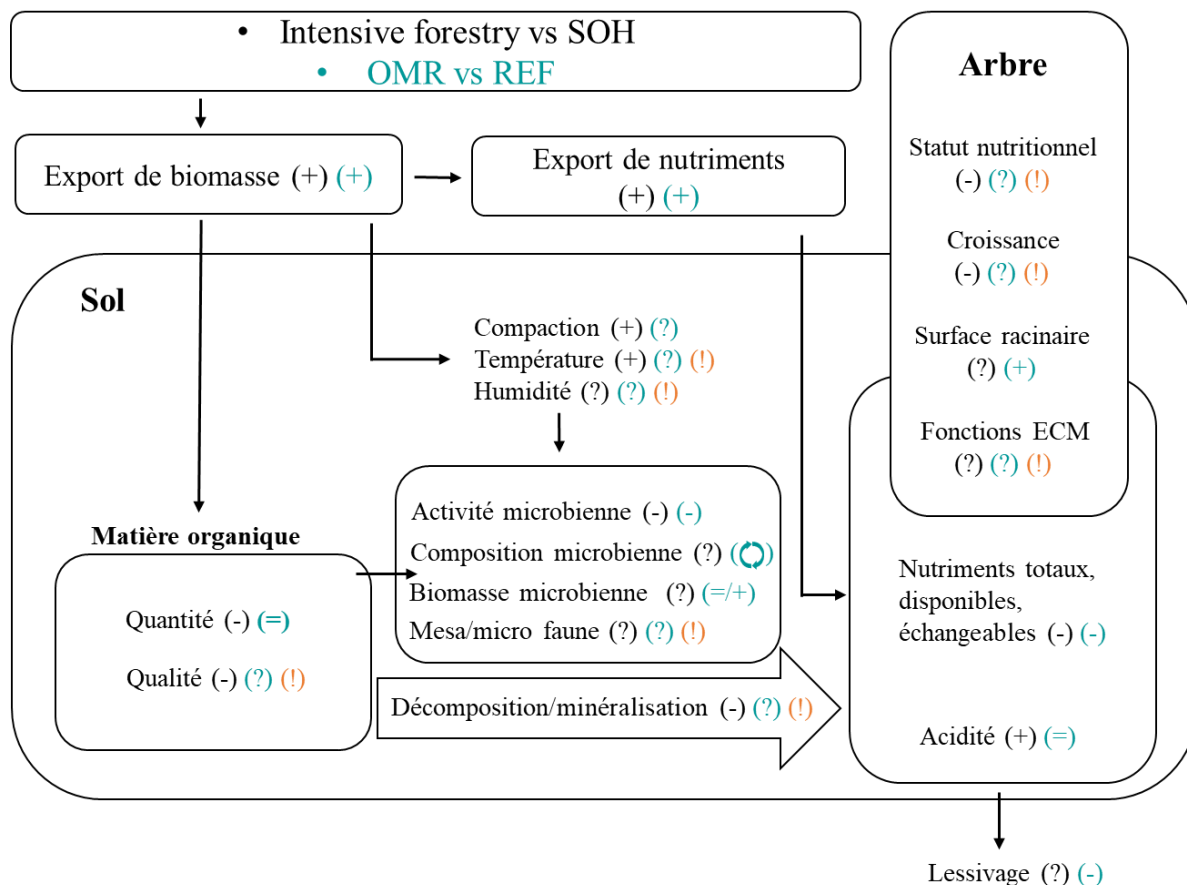


Figure 4 : Bilan des impacts de l'exportation intensive de biomasse correspondant à des pratiques sylvicoles réelles ou mimées modifié d'après Achat et al. (2015). Le type d'effet est indiqué par les signes suivants : négatif par (-), absence d'effet (=), effet positif par (+), absence de résultat par (?), résultats en cours d'acquisition et/ou d'analyse sur le réseau expérimental MOS par (!). Les signes de couleur noire indiquent les résultats d'Achat et al. (2015), les signes de couleur bleue indiquent des résultats issus du travail de recherche présentés dans cette thèse. Les signes de couleur orange indiquent des résultats en cours d'acquisition et/ou d'analyse sur le réseau expérimental MOS.

Malgré de récentes études menées sur les effets de l'intensification des pratiques sylvicoles, les potentiels impacts sur les biomasses microbiennes restaient peu étudiés. Au cours de cette thèse, nous avons mis en évidence que les biomasses microbiennes des sols semblent peu impactées par le retrait de matière organique voire tendent à augmenter dans les placettes manipulées. Cependant, la structure des communautés microbiennes change fortement en réponse au retrait de matière organique ce qui n'avait pas encore été décrit dans la littérature. En effet, si les résultats obtenus sur le réseau expérimental LTSP ont montré des changements drastiques des communautés microbiennes entre les traitements d'exportation intensive de matière organique et les placettes témoins (Hartmann et al., 2009; Hartmann et al., 2012; Wilhelm et al., 2017), la différence d'âge des forêts des placettes de retrait de matière organique et témoin semble un facteur cofondant extrêmement problématique dans ces études (Pennanen et al., 1999; Banning et al., 2011; Hagenbo et al., 2018). L'absence de différence de structure des communautés microbiennes entre le traitement de récolte d'arbre entier et d'exportation de troncs uniquement

suggère que la structure des communautés microbiennes est résiliente à l'exportation intensive de matière organique après une rotation. D'après nos résultats nous pouvons penser que la structure communautés microbiennes sera affectée par les pratiques sylvicoles à moyen ou long terme durant la première rotation ou à partir des rotations suivantes en forêt tempérée de hêtre. Nous menons actuellement une approche d'analyse de co-occurrence afin d'étudier les effets potentiels du retrait de matière organique sur la structure des réseaux de corrélations bactériens et fongiques qui semblent généralement sensibles aux perturbations (de Vries et al., 2018). Les effets du retrait de la matière organique sur la faune tellurique, qui joue un rôle clé dans le cycle du carbone et des nutriments des écosystèmes forestiers (Pant et al., 2017), sont étudiés par le laboratoire Ecodiv (Université de Rouen) dans le cadre de la thèse de M. François Elie. Les résultats de ce travail de recherche, associés à nos résultats devraient fournir une vue exhaustive de la réponse de l'édaphon au retrait de matière organique. Enfin, dans l'étude réalisée par Achat et al. (2015), les fûts de dessidus composent uniquement 20% des sites expérimentaux de la méta-analyse. Nos résultats ont permis de mettre en évidence les effets du retrait de matière organique sur les propriétés des sols et la diversité taxonomique et fonctionnelle des communautés bactériennes et fongiques des forêts tempérées de hêtre qui restaient peu étudiées.

2. Les limites de l'exportation artificielle de matière organique

La question de la comparabilité des effets de l'exportation artificielle de matière organique aux pratiques sylvicoles intensives comme la récolte d'arbre entier, reste entière. La principale limite du retrait artificiel de matière organique est qu'il engendre un sol nu. Ce sol sans litière ne correspond pas au sol des traitements d'exportation d'arbre entier qui possède une litière. On peut donc imaginer des effets indirects dus à l'exportation des litières sur l'humidité, la compaction et la température des sols qui sont différents des effets mesurés sur les placettes de récolte d'arbre entier (Achat et al., 2015). Ces trois paramètres affectent généralement significativement les communautés microbiennes (Zogg et al., 1997 ; Ponder et al., 2002 ; Fierer et al., 2003). L'une des principales limites de notre travail est l'absence de prise en compte de ces effets indirects. Pour répondre à cette limite, les sites du réseau expérimental MOS ont été équipés de sondes mesurant l'humidité et la température des sols et les résultats sont encore en cours d'analyse. Ces résultats resteront néanmoins difficiles à intégrer à nos résultats concernant les effets du retrait de matière organique sur la diversité taxonomique et fonctionnelle des communautés microbiennes. Néanmoins, les mesures des effets du retrait artificiel de matière organique sur la température et l'humidité des sols pourront être comparées

aux effets mesurés pour la récolte d'arbres entiers dans la littérature. Si les résultats sont proches, nous pourrions considérer que l'exportation artificielle de matière organique peut mimer de manière convenable la récolte d'arbre entier. Une autre limite au retrait des litières afin de mimer une exportation de matière organique est le développement massif de mousses sur les placettes manipulées qui pourrait impacter la structure des communautés microbiennes du sol de surface. Dans d'autres sites expérimentaux de retrait de matière organique, les mousses ont été éliminées (Kotroczo et al., 2014). Cette question devra être abordée rapidement dans le cadre du suivi à moyen terme des sites expérimentaux du réseau MOS. Enfin, nous n'avons pas réalisé de suivi saisonnier des effets de l'exportation artificielle de matière organique. En effet, nous avons échantillonné les sites du réseau MOS en septembre car c'est à cette période de l'année que les sites présentent le plus de jours sans litière foliaire depuis la chute des feuilles. On peut se demander si les effets du retrait de matière organique sont conservés au cours des saisons. Néanmoins, le suivi saisonnier à large échelle des sites expérimentaux du réseau MOS est en pratique difficilement réalisable. On pourrait imaginer suivre annuellement les effets du retrait de matière organique des sites les plus proches géographiquement du Centre INRA Grand-Est. L'activité microbienne de dégradation de la matière organique dépend principalement de la température et de l'humidité des sols, on peut ainsi considérer que l'automne et le printemps sont des périodes adaptés à la mesure des effets de retrait de matière organique (Zelles et al., 1992, Boerner et al., 2005; Kang et al., 2009).

3. Indiquer ou modéliser ?

L'étude des effets de l'export intensif de matière organique sur l'écosystème a deux objectifs principaux. Le premier objectif, empirique, consiste à identifier des indicateurs du dysfonctionnement précoce de l'écosystème forestiers à l'exportation intensive de matière organique. Si des indicateurs étaient mis en évidence ils pourraient être utilisés sur le terrain afin déterminer l'état d'une forêt et de limiter ou non l'exportation de résidus en fonction des résultats. Le second objectif, mécanistique, consiste en la détermination des facteurs de sensibilité des écosystèmes forestiers aux pratiques sylvicoles intensives. Ainsi, par des approches de modélisation, la détermination a priori de la sensibilité des forêts à l'exportation massive de biomasse permettrait d'adapter les pratiques sylvicoles. Il n'existe actuellement ni indicateur de dysfonctionnement des écosystèmes forestiers aux pratiques sylvicoles intensives ni modèles prédictifs de gestions. Achat et al. (2015), relève que l'incapacité à la détermination de facteurs déterminants la sensibilité des sites est dû à la grande hétérogénéité spatiale,

climatique, temporelle et de type de peuplement des études visant à comprendre les effets des pratiques sylvicoles intensives. Nos données ont permis de mettre en évidence que le retrait de matière organique affecte de manière significative les fonctions et la structure des communautés bactériennes et fongiques des sols. De plus, les effets de l'export de matière organique sont notables avant d'observer des changements des teneurs en matière organique des sols. Ces résultats suggèrent que la composante microbiologique des sols forestiers pourrait être utilisée comme bio-indicateur de dysfonctionnement. Si les approches d'étude de la diversité taxonomique demandent des technologies élevées, l'étude des fonctions de dégradation est nettement moins consommatrice en moyen. De plus, les approches enzymatiques et Biolog de caractérisations des fonctions microbiennes des sols sont apparues sensibles à l'effet des pratiques sylvicoles intensives réelles en plantation d'Eucalyptus en forêt tropicale. La détermination des facteurs de sensibilité des sites au retrait de matière organique est sûrement la piste la plus importante à explorer. Nous avons pu montrer que plus la teneur en matière organique du sol de surface est élevée et moins les fonctions microbiennes changent en réponse à l'export de matière organique. Ainsi, nous avons déterminé un facteur de sensibilité des fonctions microbiennes à l'export de matière organique. Il sera intéressant de mettre en lien ces résultats avec la croissance des ligneux en cours de mesure et d'analyse par les équipes du laboratoire SILVA (INRA). De plus, dans le cadre du réseau expérimental MOS, ces expériences pourront être étendues à deux autres essences d'intérêt économique, le chêne et le sapin de Douglas dont les placettes expérimentales sont maintenant mises en place.

4. Export de matière organique, impact sur la fertilité et le stockage de carbone des sols ?

Dans une étude récente, Kyaschenko et al. (2017) a proposé un contrôle fongique du cycle du carbone et de l'azote en forêt (Figure 5 a). En dégradant la matière organique les champignons rendent les nutriments disponibles et contribuent à la fertilité des sols. La fertilité contribue à une meilleure croissance des ligneux et une plus grande production de matière organique. Ce fonctionnement hypothétique en boucle pourrait donc être impacté par les pratiques sylvicoles intensives. En effet, nous avons observé un changement drastique des communautés fongiques. En réponse au retrait de matière organique, l'abondance relative des champignons ectomycorhiziens augmentent significativement dans le sol de surface. En changeant la balance entre champignons saprotrophes et ectomycorhiziens des sols, l'exportation intensive de matière organique pourrait affecter cette boucle, notamment si comme proposé par Gadgil and Gadgil (1971) et récemment par Fernandez and Kennedy (2016), la balance entre champignons saprotrophes et ectomycorhiziens conditionne la dégradation de la matière organique des sols. Les effets du changement des communautés fongiques sur le cycle du carbone et des nutriments pourraient ainsi dépendre des capacités de dégradation des champignons.

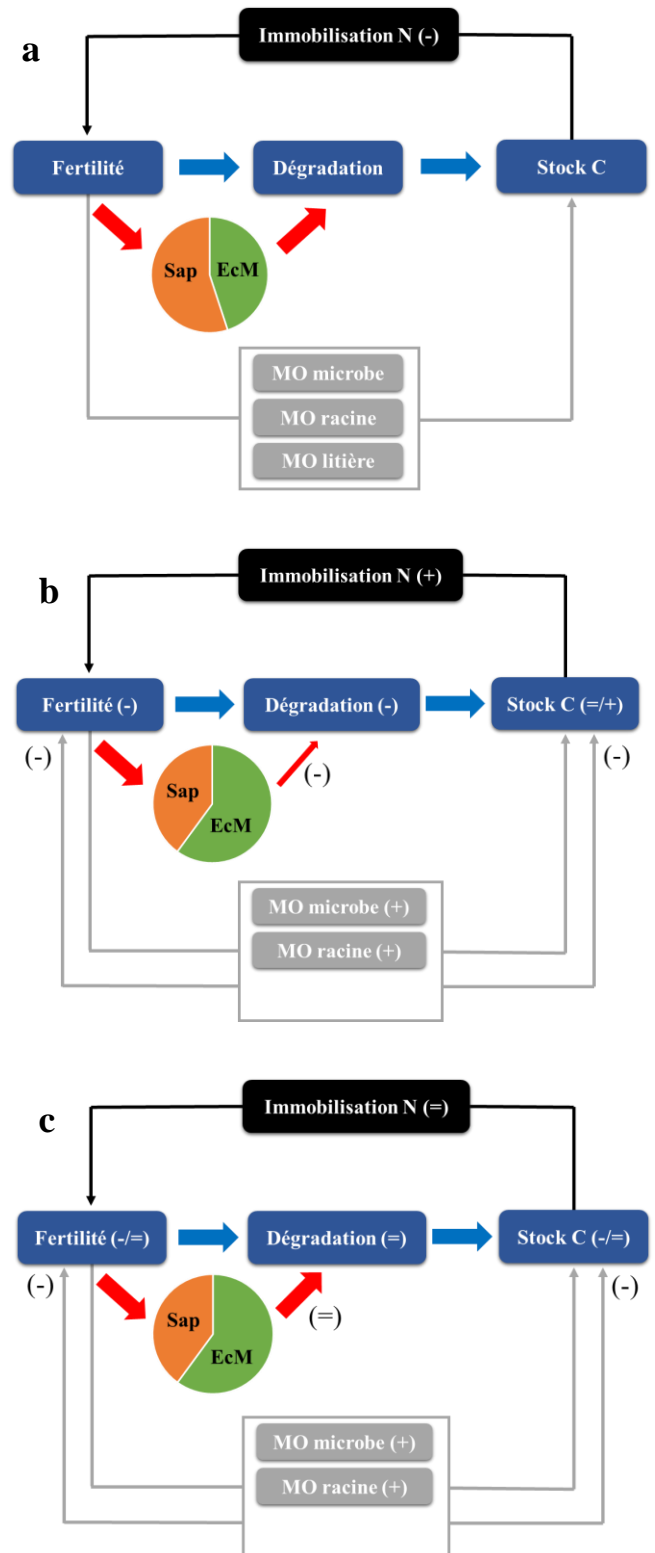


Figure 5 Rôle des champignons dans le cycle du carbone et des nutriments en forêt modifié d'après Kyaschenko et al. 2017. (a) fonctionnement forestier normal (b) réponse au retrait de MO sans capacité de dégradation de la MO par les champignons EcM (c) réponse au retrait de MO avec capacité de dégradation de la MO par les champignons EcM. EcM = ectomycorhiziens, Sap = saprotrophes ; MO = matière organique

Dans le cas où les champignons ectomycorhiziens ne posséderaient pas la capacité de dégrader la matière organique, on pourrait observer une diminution de la dégradation de la matière organique des sols d'origine microbienne ou racinaire, ainsi qu'une stabilisation des stocks de carbone et une diminution de la fertilité des sols dû à une augmentation de l'immobilisation des nutriments dans la matière organique et à l'exportation des nutriments des litières exportées (Figure 5b). Dans le cas où les champignons ectomycorhiziens posséderaient des capacités de dégradation de la matière organique, la vitesse de dégradation serait maintenue, les stocks de carbone seraient plutôt impactés négativement et la fertilité serait finalement non impactée ou diminuerait à la suite de l'exportation des nutriments contenu dans les litières (Figure 5c). Dans les deux cas envisagés ici, l'export intensif de matière organique semble avoir des effets opposés sur la fertilité des sols et les capacités de stockage de C.

Après trois ans d'export intensif de matière organique, la teneur en matière organique des sols ne diminue pas significativement. De plus, les fonctions microbiennes principalement fongiques de mobilisation de nutriments (P et N) ont drastiquement chuté en réponse au retrait des rémanents. Les teneurs phosphore ont également diminué dans les placettes où la matière organique est manipulée. Ces résultats suggèrent que l'exportation intensive de matière organique a des effets proches de l'hypothèse (b) ce qui présuppose que les champignons ectomycorhiziens ne disposent pas de capacités de dégradation majeure dans les sites étudiés (Figure 5b). Néanmoins, les approches descriptives corrélatives montrent leur limite concernant l'étude des fonctions de dégradation des champignons ectomycorhiziens (Kyaschenko et al., 2017). C'est pourquoi nous avons conduit un suivi des fonctions de dégradation des cortèges ectomycorhiziens associés aux ligneux du réseau MOS (résultats non montrés). Nous avons également suivi le statut nutritif de l'arbre par la mesure des composés carbonés et azotés non structuraux (résultats non montrés). Ces résultats sont en cours d'analyse et devraient nous permettre de déterminer si les champignons ectomycorhiziens répondent à l'exportation de matière organique en augmentant leur potentiel de dégradation de la matière organique et également de connaître le statut nutritif de l'arbre. Nous avons également cherché à déterminer *in vitro*, les capacités de dégradation de la matière organique de champignons ectomycorhiziens.

5. Les champignons ectomycorrhiziens, potentiels décomposeurs ?

Une étude génomique que nous conduisons en collaboration avec Francis Martin (DR INRA Grand-Est Nancy) et Shingo Myauchi (Chercheur postdoctorant, INRA Grand-Est Nancy) nous a permis de conclure que si les champignons ectomycorrhiziens ont perdu beaucoup de gènes codant des fonctions impliquées dans la dégradation des parois végétale, ils conservent un nombre important de gènes codant des fonctions impliquées dans la dégradation des parois microbiennes et notamment fongiques (résultats non montrés). Ainsi nous avons émis l'hypothèse que les champignons ectomycorrhiziens pourraient dégrader la nécromasse fongique et également un des composants principaux de la nécromasse fongique, la chitine. Nous avons démontré que *Paxillus involutus*, un champignon ectomycorhizien, est capable de mobiliser et de transférer de l'azote provenant de nécromasse fongique à son hôte végétal. Nous avons également déterminé que certains champignons ectomycorhiziens sont capables, en culture pure, de mobiliser de l'azote organique provenant de chitine fongique. Ainsi, les protéines ne représentent plus les seuls polymères dégradables par les champignons ectomycorhiziens. Cependant on peut s'interroger sur le fait que si les champignons ectomycorhiziens possèdent des activités de dégradation de la matière organique et notamment la chitine, pourquoi mesure t'on une chute si importante des activités chitinolytiques des sols en réponse à l'exportation de matière organique sur les sites du réseau experimental MOS ? Les espèces ectomycorhiziennes capables de dégrader la chitine d'après nos résultats sont très peu abondantes dans les sols des forêts tempérées de hêtre du réseau MOS. Le genre *Russula*, domine très nettement les abondances relatives des horizons organiques et minéraux des sites du réseau MOS. Malheureusement ce genre fongique est difficile à isoler et à une croissance très limitée en laboratoire ce qui rend l'évaluation de son potentiel chitinolytique difficile. Les profils enzymatiques des ectomycorhizes de *Russula in situ* permettent difficilement de déterminer le potentiel de dégradation de la matière organique des champignons du genre *Russula* (Buée et al., 2007 ; Pritsch et al., 2011). Des génomes de champignons du genre *Russula* sont en cours de séquençage, et leur étude permettra de déterminer si les russules possèdent ou non le potentiel génomique de dégradation de la chitine (Looney et al., 2018). On peut ainsi supposer que pour des forêts présentant des communautés fongiques ectomycorhiziennes ayant un potentiel de dégradation de la matière organique important, les impacts de l'exportation intensive de rémanents et par extanction des pratiques sylvicoles intensives sur le fonctionnement biologique des sols pourraient être différents.

6. Références

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CONCLUSION GENERALE

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L'étude des effets réels ou mimés des pratiques sylvicoles intensives a permis de mettre en évidence une diminution des fonctions microbiennes telluriques de dégradation de la matière organique. De plus, à large échelle et en forêt tempérée, les communautés fongiques et bactériennes changent en réponse au retrait de matière organique. Ce changement se matérialise par une diminution de l'abondance relative des champignons saprotrophes et des bactéries copiotrophes et une augmentation des champignons ectomycorhiziens et des bactéries oligotrophes dans les sols des placettes où la matière organique est retirée. Le changement des communautés microbiennes en réponse à l'exportation de matière organique pourrait impacter le cycle du carbone et des nutriments des forêts tempérées. Afin de mieux comprendre ces possibles effets nous avons étudié le potentiel de dégradation de la matière organique des champignons ectomycorhiziens. Nous avons démontré que certains champignons ectomycorhiziens sont capables de mobiliser de l'azote organique provenant de nécromasse et de chitine fongique. Ces résultats, associés aux autres études menées sur le réseau expérimental MOS, nous permettront de construire des modèles de prédiction des effets négatifs des pratiques sylvicoles intensives sur le fonctionnement de l'écosystème forestier à destination des sylviculteurs afin d'adapter la gestion sylvicole pour une exploitation durable.

Résumé:

En Europe, le bois est la première source d'énergie renouvelable. La transition énergétique se traduit par une intensification de l'exploitation des forêts. L'effet de ces pratiques sylvicoles sur les communautés microbiennes du sol est encore peu étudié. Au cours de ma thèse, j'ai évalué les conséquences d'une manipulation artificielle de matière organique en forêt tempérée sur la diversité fonctionnelle et taxonomique des communautés bactériennes et fongiques telluriques dans six sites expérimentaux (réseau expérimental MOS). Parallèlement, une caractérisation fonctionnelle des communautés microbiennes a également été réalisée dans un contexte proche des réalités de l'intensification des pratiques sylvicoles sous climat tropical en plantation d'Eucalyptus. Si certains descripteurs fonctionnels de la dégradation de la matière organique sont particulièrement informatifs, les activités microbiennes de dégradation de la chitine, polymère azoté des arthropodes et champignons, sont apparues très sensibles au retrait de matière organique. C'est pourquoi, par des approches de génomiques comparatives, nous avons cherché à estimer le potentiel chitinolytique des différentes guildes fongiques des sols. En conditions contrôlées, nous avons ensuite quantifié les capacités potentielles de mobilisation et de transfert du carbone et de l'azote, à partir d'une matière organique microbienne riche en chitine, par un champignon ectomycorhizien en symbiose avec son hôte. Enfin, la généralité des fonctions chitinolytiques d'un plus large spectre d'espèces fongiques ectomycorhiziennes a été évaluée par le couplage d'approches enzymatiques et isotopiques. L'ensemble de nos résultats met en lumière le rôle significatif des champignons ectomycorhiziens dans la mobilisation du carbone et de l'azote à partir de certaines formes de matière organique, et la nécessité de prendre en compte le compartiment microbien dans les études d'impact des pratiques sylvicoles.

Mots clés : communauté microbienne, fonctions microbiennes, champignons, bactéries, champignons ectomycorhiziens, matière organique, forêt tempérée, chitine.

Abstract:

One of the main usages of wood in Europe is renewable energy supply that implies intensification of forest management to respond to this increasing demand. However, the impact of intense forestry practices on soil microbial communities remains poorly investigated. In the frame of my PhD thesis, I evaluated effects of artificial organic matter removal on functional and taxonomical diversity of soil bacterial and fungal communities in temperate forest, using six experimental sites across France (INRA MOS experimental network). In parallel, I also characterised impact of intensified forest management practices on functional microbial communities in tropical plantation of Eucalyptus trees. This work permitted to identify several sensitive functional indicators of organic matter degradation. Notably, the degradation of chitin – a nitrogen polymer main component of arthropods and fungal cell walls – was revealed to be particularly sensitive to organic matter removal. Genomics and enzymatic approaches were then used to estimate chitinolytic potentials of the different genera of soil fungi. In controlled conditions, we were able to quantify ectomycorrhizal fungus carbon and nitrogen mobilisation and transfer capacities from chitin enriched organic matter to its host during symbiotic interaction. Finally, we evaluated chitinolytic functions of ectomycorrhizal fungi at large scale by combining enzymatic and isotopic approaches. Taken together, the results acquired in the frame of my PhD thesis, illustrate the significant role of ectomycorrhizal fungi in carbon and nitrogen mobilisation from organic matter. We particularly highlight that microbial compartment in soil must be considered in studies of forest management practices.

Keywords: microbial community, microbial functions, fungi, bacteria, ectomycorrhizal fungi, organic matter, temperate forest, chitin.