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Impact of manganese concentration on litter decomposition

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Thèse présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

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« Celui qui est le premier à voir l'Amérique. Sur chaque bateau il y en a un. Et il ne faut pas croire que c'est le hasard, non … ni même une question de bonne vue, c'est le destin, ça. Ces types-là, depuis toujours, dans leur vie, ils avaient cet instant-là d'écrit. Même tout petits, si tu les regardais dans les yeux, en regardant bien, tu la voyais déjà, l'Amérique, elle était là, prête à bondir, à remonter le long des nerfs ou du sang ou je ne sais quoi, et puis, dans ce cri (il crie), l'AMERIQUE, elle était déjà là, dans ces yeux, ces yeux d'enfant, déjà là toute entière, l'Amérique.

Là, qui attendait.

Celui qui m'a appris ça, c'est Danny Boodman T.D. Lemon Novecento, le plus grand pianiste qui ait jamais joué sur l'Océan. Dans les yeux des gens, on voit ce qu'ils verront, pas ce qu'ils ont vu. Il disait ça : ce qu'ils verront. »

Extrait de « Novecento: pianiste » d'A. Baricco

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S'embarquer dans la réalisation d'une thèse, c'est partir en voyage vers une destination pas toujours connue au départ …
Me voici arrivée à destination !

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Contents

General introduction & Thesis outline

General introduction 6
"State of art" 6
General objective 7
Thesis outline 8

Part I - General overview

Overview outline 14

I. Litter decomposition in temperate ecosystems 15

1. Development of issues contextualizing litter decomposition studies 15
2. Key developments in litter decomposition studies 19
2.1. What is decomposition? 19
2.2. What controls it? 22

II. Lignin degradation 25

1. Lignins 25
2. Ligninolysis 29
3. Which factors influence ligninolysis? 33

III. Review of papers studying the impact of manganese on litter decomposition 35

IV. Several functions of manganese in the organic matter decomposition process 42

1. Forms and main functions of manganese 42
2. Involvement of manganese in N-transformations 45
3. Involvement of manganese in microbial activity 48
4. Involvement of manganese in ligninolysis 50

Part II - Impact of tree species on litter decomposition: focus on manganese concentration

I. Summary of the study 55
II. Tree species have different ability to recycle manganese. 56
III. A simple estimator of relative nitrification 57
IV. Correlations with manganese concentration 58

- - - Photoboard I - - -

Part III - Effect of manganese addition on carbon release from forest floor horizons

Abstract 64
Résumé 65
I. Introduction 66
II. Materials and methods 68
1. Study sites 68
2. Sampling and analysis of forest floors 69
3. Incubation, chemical analyses of water extracts, and C-mineralization 69
III. Results and Discussion 71
1. Forest floor characteristics 71
2. Effects of Mn addition on C release 75
3. Conclusion 79

- - - Photoboard II - - -

Part IV, Chapter 1 - Influence of manganese on beech leaf litter decomposition during *in situ* incubation

Abstract 82
I. Introduction 83
II. Materials and methods 85
1. Pre-treatment of beech leaves 85
2. Litterbag experiment (Photoboard III) 87
3. Chemical analysis of litters and leachates 89
4. Statistical analysis 90

III. Results 92

1. Composition of beech leaves 92
2. Assessment of decomposition rate 94
3. Litter quality changes 97
4. Leachates composition 98

IV. DISCUSSION 100

1. Mn concentration in leaves 100
2. Impact of Mn on litter decomposition 100
3. Impact of Mn on leachate properties 101
4. Conclusions 102

- - - Photoboard III - - -

Part IV, Chapter 2 - Influence of manganese on carbon released by decomposing beech leaf litters: a microcosm experiment

Abstract 108

I. Introduction 109

II. Materials and methods 111

1. Pre-treatment of beech leaves 111
2. Laboratory incubation in microcosms 111
3. Chemical analysis of litter 112
4. Analysis of branch litter leachates 114
5. Statistical analysis 114

III. Results and discussion 115

1. CO_2 release 115
2. DOC release 120
3. CO_2 and DOC releases 121
4. Change in litter decomposition during the incubation period 123
5. Conclusions 125

Part IV, Chapter 3 - Manganese effect on lignin degradation degree in beech leaf litters

Abstract 128

I. Introduction 129

II. Materials and methods 131

1. Leaf litter samples 131
2. Leaf litter analysis 133

III. Results and discussion 136

1. Effect of manganese concentration on lignin degradation 136
2. Effect of manganese concentration on lignin characteristics during degradation 138
3. Relationships between litter properties (PCA) 141
4. Relationships between litter samples 143
5. Conclusion 144

General discussion & Conclusions

Why were we interested in the impact of manganese concentration on forest litter decomposition? 146

What was our specific scientific question? 146

Which experiments were carried out to answer to this scientific question? ... 147

What did these differences of experimental characteristics involve? 149

Focus on some results 151

Perspectives? 154

Appendices

Appendix 1
Influence of tree species on carbon and nitrogen transformation patterns in forest floor profiles 156

Appendix 2
Experimental methods determining lignin quantity and quality 183

List of relate communications 189

References

General introduction
&
Thesis outline

General introduction

Litter decomposition is a crucial process in the nutrient cycles of most terrestrial ecosystems and is of particular relevance for tree growth. Simultaneously, litter decomposition affects the processes in underlying soils through the release of various elements (Berthelin *et al.*, 1990; Guggenberger and Kaiser, 1998). Moreover, decomposition of dead organic matter controls the rate of carbon released to the atmosphere (Heim and Frey, 2004) and stabilized in soil (Lal, 2005) and as such, strongly impacts the global carbon budgets on the Earth's surface.

"State of art"

Since the scientists were interested in litter decomposition, they have among others focused on the use of different litter quality indexes as litter decay predictors (C/N ratio (Anderson, 1973; Berg and Staaf, 1980), essential nutrients such as N and P (Prescott *et al.*, 2000; Vesterdal and Raulund-Rasmussen, 2002), lignin concentration (Melillo *et al.*, 1982; Meetenmeyer, 1978), *etc*).

Recently, manganese concentration has begun to receive attention in litter decomposition studies given its implication in the catalytic cycle of the manganese peroxidase, the main enzyme of the lignin degrading system (Hammel, 1997; Hofrichter, 2002). Indeed, several researchers put in light empirical relations between initial Mn concentration in litter and parameters related to litter decomposition. For example:

- Berg *et al*. (1996) used the data of 20 litter types incubated in eight different ecosystems and showed a positive correlation between Mn concentrations and the levels of limit values (R^2 = 0.372, n=25, $p < 0.0010$).
- In an oak (*Quercus robur* L.) litters study, Davey *et al*. (2007) found that the most significant correlation between initial nutrient values and annual mass loss (k) was the initial Mn concentration in litter (R^2, n=20, p=0.0070).

- Berg *et al.* (2007) showed a statistically significant linear relationship between annual mass loss (*k*) of foliar litters in the late stage of decomposition and Mn concentration in the litters.
- Berg *et al.* (2010) concluded that Mn concentration may predict the fraction of extremely slowly decomposing fraction of litter for pine and that Mn concentration may be used to estimate the accumulation of humus and carbon sequestration from pine litter.

However, the published studies do not seem to be unanimous about the exact effect of Mn on lignin degradation. The relationship is not equivocal and seems to be dependent on early stage? on late stage? on litter species? on critical concentration of Mn in litter? It nevertheless seems as though there was a significant relation between Mn concentration and parameters related to litter decomposition.

General objective

Therefore, this thesis focuses on the impact of Mn concentration on litter decomposition. The approach consists of using methods of litter incubation allowing the control of confounding effects. We assessed litter decomposition by analysis of the composition of decomposing organic materials and of water leachates.

Thesis outline

Figure A gives an overview of the thesis outline. The thesis is structured in four parts. Most of the parts/chapters were constructed as a scientific publication with its own introduction and conclusion.

➲ Part I – General overview

This part gives a general overview of the current knowledge on litter decomposition in temperate ecosystems, including lignin degradation, gives a review of the papers studying impact of manganese on litter decomposition and provides the functions of manganese in the organic matter decomposition.

➲ Part II – Impact of tree species on litter decomposition: focus on manganese concentration.

This part focuses on manganese concentration in forest floors of four tree species in the scope of our study entitled "*Influence of tree species on carbon and nitrogen transformation patterns in forest floor profiles*" (Trum *et al.*, 2011 b). This exploring part allows us to highlight relationships between manganese concentration in forest floor horizons and (i) carbon mineralization and (ii) nitrification degree.

➲ Part III – Effect of manganese addition on carbon release from forest floor horizons.

This part reports the results of carbon release (CO_2 and DOC) from three horizons of two beech (*Fagus sylvatica* L.) forest floors (moder and mor) separately incubated in laboratory with or without Mn^{2+} addition (through $MnSO_4$ aspersion). The main conclusions of this experiment are that (i) Mn effect depended on the horizon and on the forest floor and that (ii) the availability of the added Mn^{2+} was limited by the environmental conditions (pe-pH).

➲ Part IV

Because the availability of Mn^{2+} added by aspersion was limited by the environmental conditions (pe-pH) (Part III), we developed another method to dispose of litter materials of different manganese concentrations. This part is centred on the incubation of beech (*Fagus sylvatica* L.) leaf litters experimentally enriched in manganese. Part IV is divided in three chapters:

- ➲ Chapter 1 – Influence of manganese on beech leaf litter decomposition during *in situ* incubation.

In this chapter, beech leaf litters experimentally enriched in manganese were incubated *in situ* during 500 days using a litterbags experimental design. The results concern the decay rate, the carbon and nitrogen releases.

- ➲ Chapter 2 – Influence of manganese on carbon released by beech leaf litter: a microcosm experiment.

Simultaneously, beech leaf litters experimentally enriched in manganese were incubated in laboratory during 300 days

- ➲ Chapter 3 – Manganese effect on lignin degradation degree in beech leaf litters.

As the effect of manganese on litter decomposition are probably due to its implication on lignin degradation, this chapter presents the results of lignin phenolic compounds obtained by chemolytic analyses of lignin (cupric oxide oxidation method, Hedges and Ertel, 1982) before and after the both incubations. Indexes computed from these compounds provide excellent means of evaluating the quality and the degradation state of lignin (Thevenot *et al.*, 2010).

The *general conclusions and perspectives* are given at the end of this thesis.

Figure A: Schematic presentation of the thesis outline.

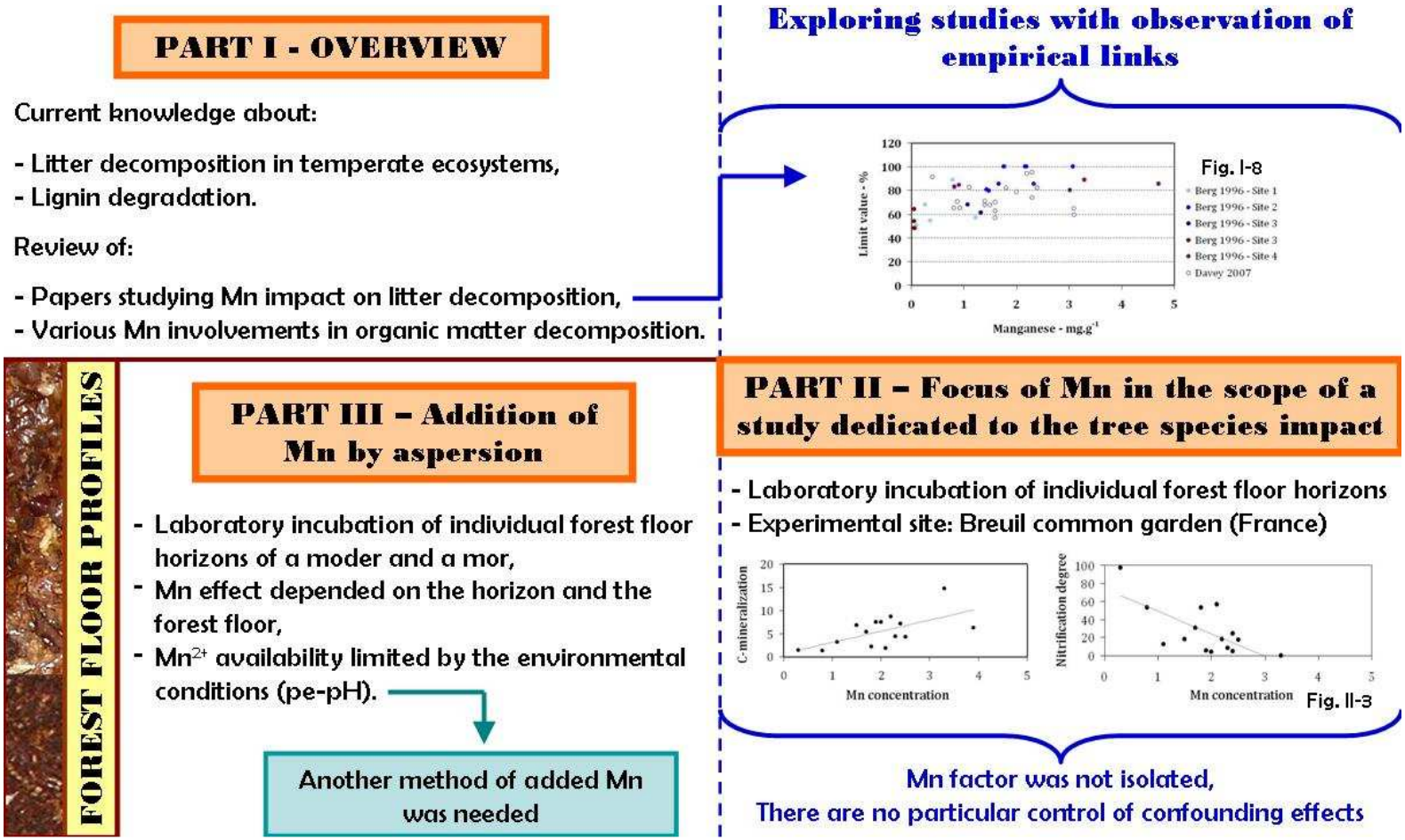
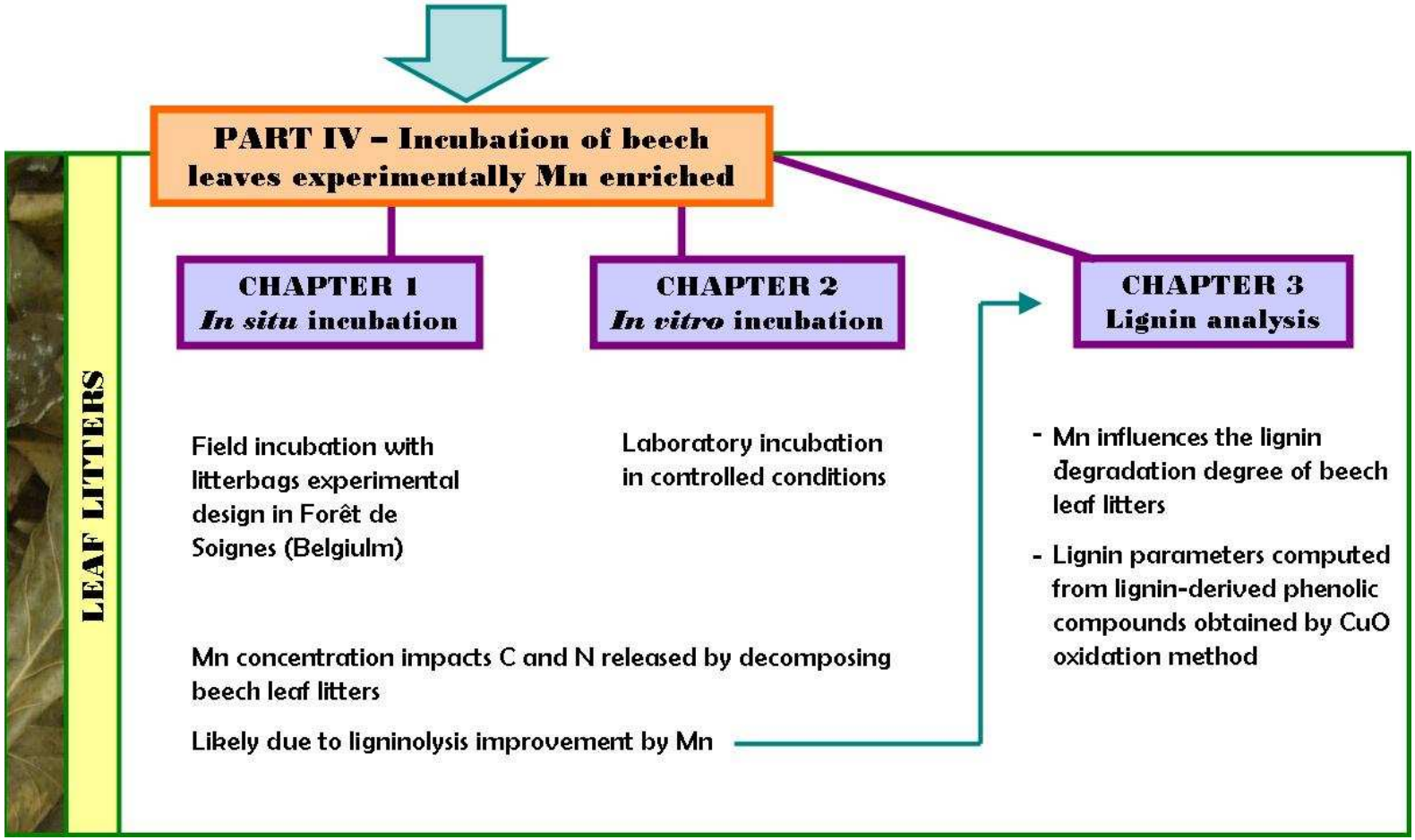


Figure A: Schematic presentation of the thesis outline.



Part I

General overview

Overview outline

This thesis is a study of the impact of manganese concentration on litter decomposition. This overview begins by surveying the current state of knowledge on litter decomposition, which is of primary importance for the sustainability of production in forest ecosystems.

➲ I. Litter decomposition in temperate ecosystems

Several factors influence litter decomposition. The most important rate-retarding factor among them is considered to be lignin concentration. Point II of this overview focuses on lignin structure and on the ligninolytic process.

➲ II. Lignin degradation

Lignin degradation is controlled by various factors (e.g. N, Mn, pH and O_2). Several recent studies have highlighted a positive correlation between the rate of forest floor decomposition and manganese concentration in litterfall given the implication of manganese in the lignin degrading system. Point III of this overview supplies a critical review of these studies.

➲ III. Review of papers studying the impact of manganese on litter decomposition

Manganese is the tenth most abundant element in the Earth's crust and occurs in many forms depending on pe-pH conditions. Manganese plays a part in numerous plant and soil processes, mainly as a Lewis acid or as an oxidation catalyst. Hence, point IV aims to summarize those processes linked to organic matter decomposition where manganese may have an effect.

➲ IV. Several functions of manganese in the organic matter decomposition process.

I. Litter decomposition in temperate ecosystems

Litter decomposition is a key process in the nutrient cycles of most terrestrial ecosystems and is of particular relevance for tree growth, because decomposing litter (including root litter) is a significant source of nutrients, especially nitrogen (Prescott *et al*., 2000).
Simultaneously, it affects soil processes through the release of inorganic and organic proton donors (Berthelin *et al*., 1990; Guggenberger and Kaiser, 1998). Moreover, decomposition of dead organic matter controls the rate of carbon released to the atmosphere (Heim and Frey, 2004) and stabilized in soil (Lal, 2005) and as such, strongly impacts the global carbon budget on the Earth's surface

1. Development of issues contextualizing litter decomposition studies

Research into the process of litter decomposition has grown steadily since the development in the 1960s of the litterbag technique, which allows us to measure mass loss and to estimate decomposition rates (through calculation of the decomposition constant or *k* value), as well as to compare rates between sites, litter types and treatments of many kinds (Prescott, 2010). As the forest floor is rapidly affected by modifications of ecosystem parameters (Ste-Marie and Paré, 1999), it is one of the most reactive compartments of the forest soil; and as such, it has been used as a diagnostic tool for ecosystem functioning (Vesterdal and Raulund-Rasmussen, 1998).

This increased demand for knowledge can be seen clearly in Figure I-1, which shows the number of published studies available through Web of Science in 5-year periods from 1970 to today. In contrast to the slow rate of 20 papers per year in the 1980s, there is now more than one paper on litter decomposition appearing each day (Prescott, 2010), which makes litter decomposition the most frequently studied process in temperate ecosystems.

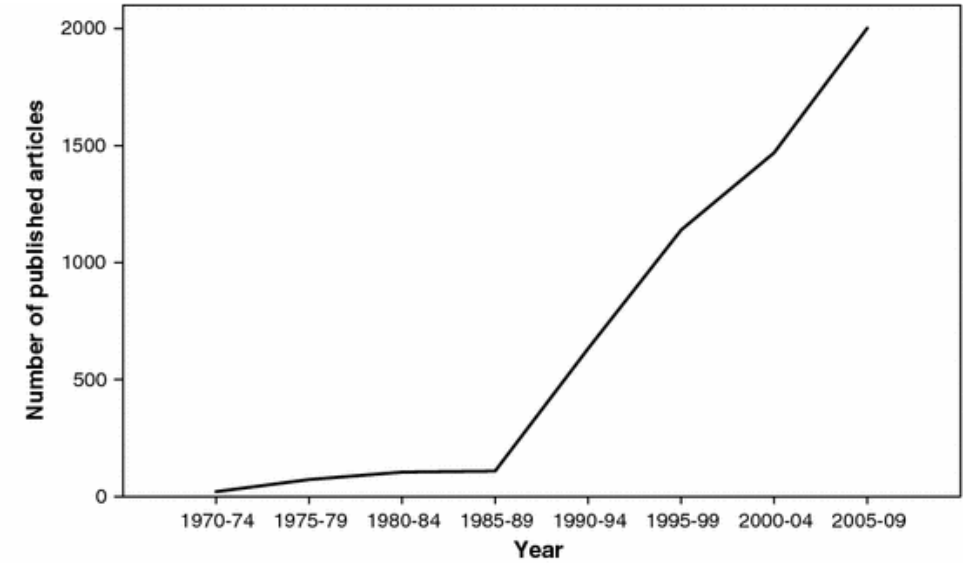


Figure I-1: Number of published studies on litter decomposition in 5-year periods from 1970 to October 20, 2009 as searched through Web of Science (from Prescott, 2010).

The environmental issues contextualizing research questions in litter decomposition studies have changed over time:

- Soil acidification due to tree species substitution

In order to increase wood production, forestry was intensified in the post-war years with a species change from hardwood to more productive conifers (Augusto *et al.*, 1998). Tree species substitution led to notable modifications of the ecosystem, so it was legitimate to look for their short- and long-term impact on soil fertility.

- N-deposition

Human activities (fertilizer consumption, fossil-fuel emissions, biomass burning, *etc*) have more than doubled inputs of nitrogen to terrestrial ecosystems worldwide (Matson *et al.*, 2002). As a result, the potential for N-deposition to affect temperate ecosystems has been widely studied (e.g. N-mineralization, nitrification, nitrate loss and net primary production) (Aber *et al.*, 1998).

- Global climate change

With the introduction of the Kyoto protocol, scientists' interests have become focused on the possible effects of the global climate change (increase in temperature, increase in CO_2 concentration in the atmosphere, *etc*) on the carbon storage, nutrient dynamics, soil chemistry and productivity of forest ecosystems (Aber, 1990).

- Greenhouse gases mitigation and carbon sequestration

Currently, the main objective of soil scientists is to develop optimal strategies to sequester more carbon in soil (Jandl *et al.*, 2007). According to Prescott (2010), the new challenge regarding litter decomposition is not to determine how to slow decomposition, but rather how to divert more carbon into humus and SOM pools rather than allowing it to decompose.

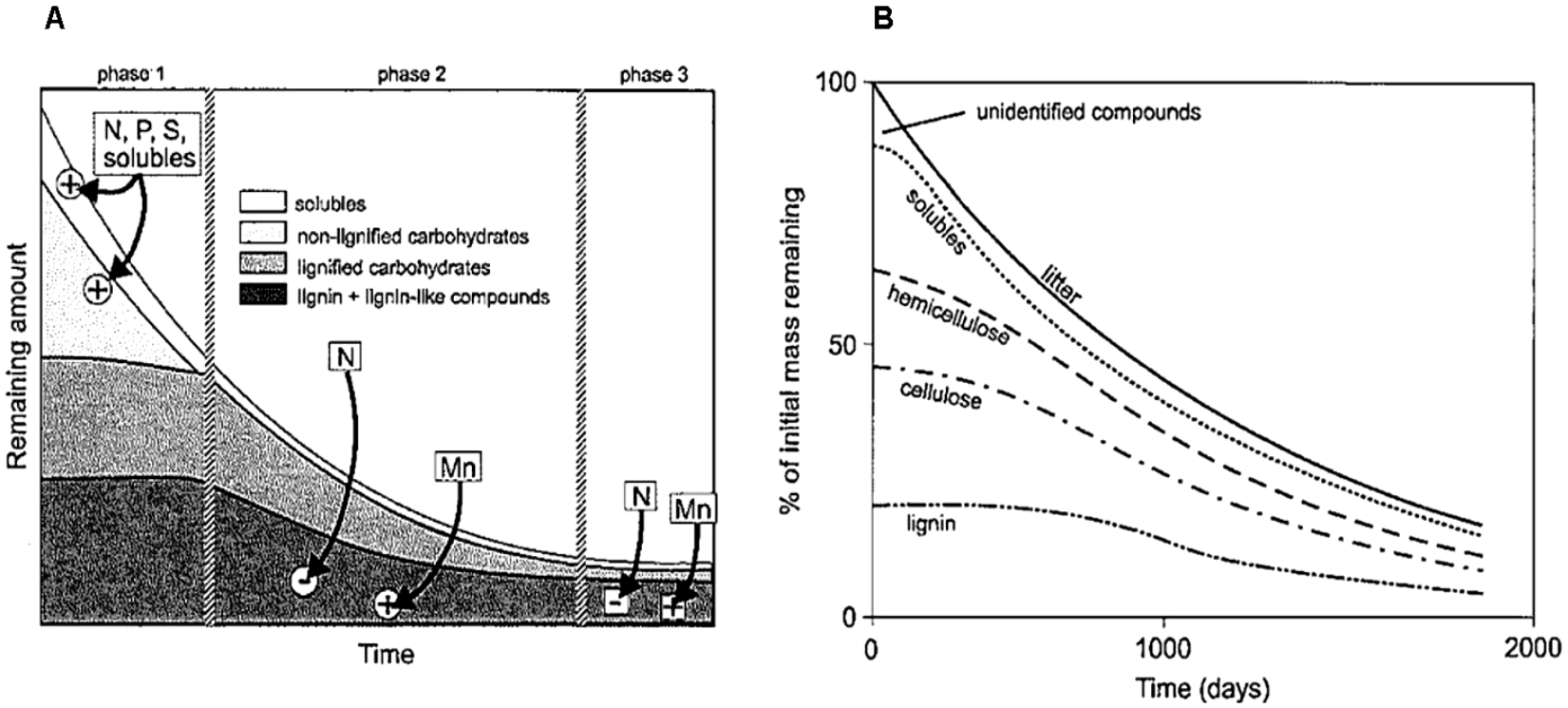


Figure I.2: (a) Model for chemical changes and rate-regulating factors during decomposition (from Berg, 1986); (b) Degradation pattern of Scots pine needle litter. Remaining amounts of litter solubles, cellulose, hemicellulose and lignin are given.

2. Key developments in litter decomposition studies

Organic matter decomposition may be defined as the physical, chemical and biological mechanisms transforming organic matter into increasingly stable forms (Berg and McClaugherty, 2008). Litter mass loss or decomposition involves two simultaneous and fundamental processes:

- The concomitant mineralization and humification of lignin, cellulose and other compounds by a succession of microorganisms,
- The leaching downwards in the soil of soluble compounds (Coûteaux, 1995).

2.1. What is decomposition?

Although the decay sequence from fresh litter to stable organic matter is continuous, Berg (1986) proposed to describe the decomposition process conceptually by a *two-phase* pattern with respect to the variation in chemical composition taking place (Figure I-2). Sometimes, a third phase is also defined as a nearly humus stage (Berg and Matzner, 1997).

o Phase I – the early stage of decomposition

Microorganisms start degrading plant litter as soon as it has fallen on the ground and been invaded by decomposers (e.g. fungal mycelium and bacteria). Those microorganisms able to use the soluble components start degrading them first and at a relatively high rate. The reason for this is that normally small molecules are more easily available to microorganisms since they may be transported directly into the cell and metabolized. There is thus no need of additional enzymes to depolymerise the larger molecules. Consequently, during the initial stage of decomposition, unprotected and monomeric compounds (labile fraction) are lost as well as some cellulose, resulting in an accumulation of lignin (Figure I-2) (Swift *et al*., 1979).

Berg (1986) suggests that concentrations of nutrients limiting the microbial activity, such as N, P and S, control this stage of decomposition (Figure I-2).

- Phase II – the late stage of decomposition

The late stage is characterized by the slow decomposition of a more recalcitrant and, thus, less biodegradable fraction related to lignin concentration (Berg and Staaf, 1980). An inverse relationship between litter mass loss and initial lignin concentration often exists, not only because lignins are recalcitrant as a consequence of their aromatic composition and structure, but also because they can physically inhibit the activity of carbohydrases by masking substrate surfaces of hemicellulose and cellulose (Melillo *et al.*, 1982).

- The concept of limit value

The decomposition process normally reaches a final stage at which it almost slopes or goes so slowly that this stage may approximately be described mathematically by an asymptotic function (Figure I-3, Eq. I-1) (Howard and Howard, 1974). The limit value is not an indication of the organic matter remaining completely undegradable. Instead, it consists of a moderately stabilized fraction decomposing very slowly (Berg *et al.*, 1996).

$$MassLoss = LV * (1-e^{-kt/LV}) \qquad \textbf{Eq I-1}$$

Where the variables are :

MassLoss is the accumulated mass loss (%)

t is the time in day

Where the parameters are :

LV is the limit value for decomposition

k is the rate of decomposition

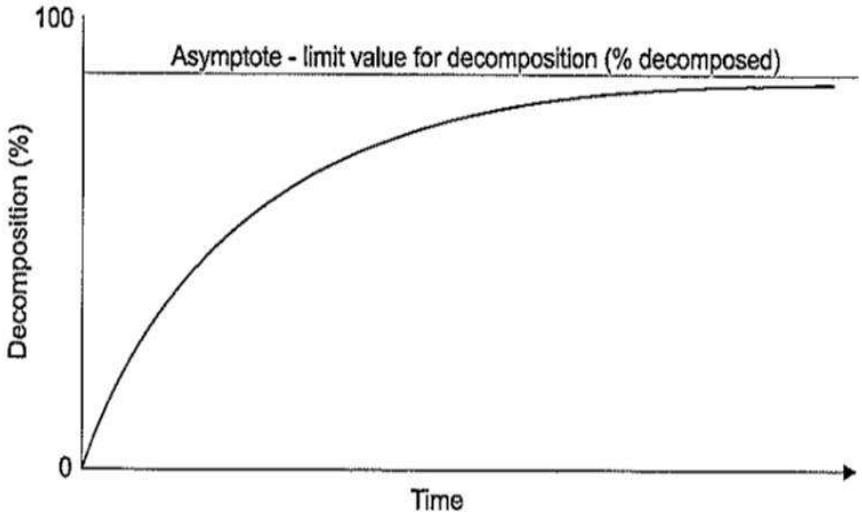


Figure I-3: Typical graph for an asymptotic function. The limit value indicates the recalcitrant fraction of the litter (Berg and Laskowski, 2006).

2.2. What controls it?

Figure I-4 summarizes the factors impacting the decomposition process. Litter decomposition rates are controlled by environmental conditions, by chemical composition of litter and by soil organisms (Cadish and Giller, 1997). These factors are not independent of each other. There are three main levels of litter decomposition control, operating in the following order: climate > litter chemistry > soil organisms (Swift *et al.*, 1979).

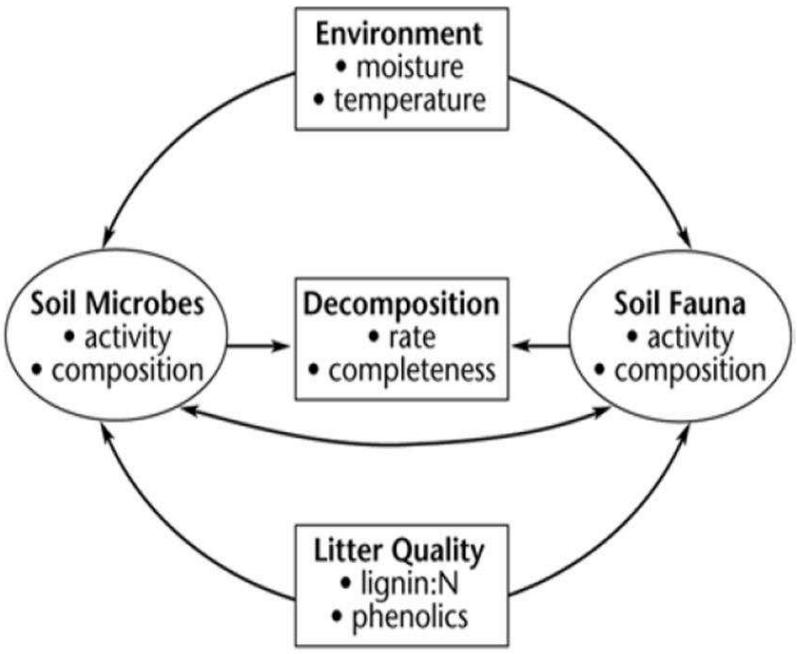


Figure I-4: Factors controlling decomposition rate and completeness (from Prescott *et al.*, 2000).

- A short retrospective (Berg and McClaugherty, 2008)

"*Already in 1929, Tenney and Waksman postulated that decomposition rates of soil organic matter are controlled by four distinct factors: (1) The chemical composition of the substrate; (2) A sufficient supply of nitrogen for the decomposer organisms; (3) The nature of the microorganisms involved; and (4) Environmental conditions, especially aeration, moisture supply, pH and temperature. As a general statement concerning factors that control decay, this is still valid more than 70 years later.*
During the intervening 70 years, we have progressed in understanding many of the details of decomposition. We have gained a deeper insight into the effects of substrate quality and environmental factors, both on local and regional scale. Studies have begun to show us more about the implication of decomposition on nutrient cycling"

- Environmental conditions (soil and climate)

It is often stated that litter decomposition is slow in cold forests (e.g. forests at high elevation or at northern latitudes), which leads to large accumulation of humus in these forests. Indeed, adequate conditions of both temperature and moisture must be present for decomposition to proceed. Measures such as actual evapotranspiration (AET) that incorporate both temperature and moisture have been used successfully to predict rates of decomposition across a wide range of climates (Meentemeyer, 1978). For example, initial lignin concentration being fixed at 25 %, the annual decomposition rate (%) was predicted to increase when the evapotranspiration (mm) increases (25 % at 400 mm *vs* 50 % at 800 mm) (Meentemeyer, 1978).

Climate determines to a large extent the distribution of soil types. Indirectly, soil type may influence decomposition via the composition of the decomposer community and the resource quality of the plant litter input. The general observation is that low-fertility soils are often characterized by accumulation of large mor-like litter layers whereas fertile soils are characterized by mull and only a minor litter layer (Raulund-Rasmussen and Vejre, 1995).

o Chemical composition of litter

The chemical composition of litter is directly linked to the tree species. Studies of tree species difference within single site have led to a belief that certain tree species tend to develop a relatively thick, acid forest floor (mor), whereas others develop a relatively thin and less acid forest floor (mull).

These differences in decomposition are attributed to variation in litter quality. Several litter properties have been identified as indicator of litter quality because they may be good predictors of litter decomposition rates in many ecosystems.

The main litter quality indexes are the following:

- Litter decomposition rates decrease with C/N ratio (Anderson, 1973; Berg and Staaf, 1980).
- Litter decomposition rates increase with nutrient concentration, particularly with N and P concentrations (Vesterdal and Raulund-Rasmussen, 2002). Microorganisms involved in litter decomposition have to rely largely on the original nutrient stocks of the decomposing tissues. This may explain why the initial N and P concentrations of a substrate exert an important influence on decomposition rate (Melillo *et al*., 1982).
- Litter decomposition rates decrease with lignin concentration (Meetenmeyer, 1978; Melillo *et al.*, 1982). Since lignin is held to be considered as a rate-retarding compound (Fogel and Cromack, 1977), lignin concentration remains a privileged litter quality index and is considered to be the major factor in litter decay.

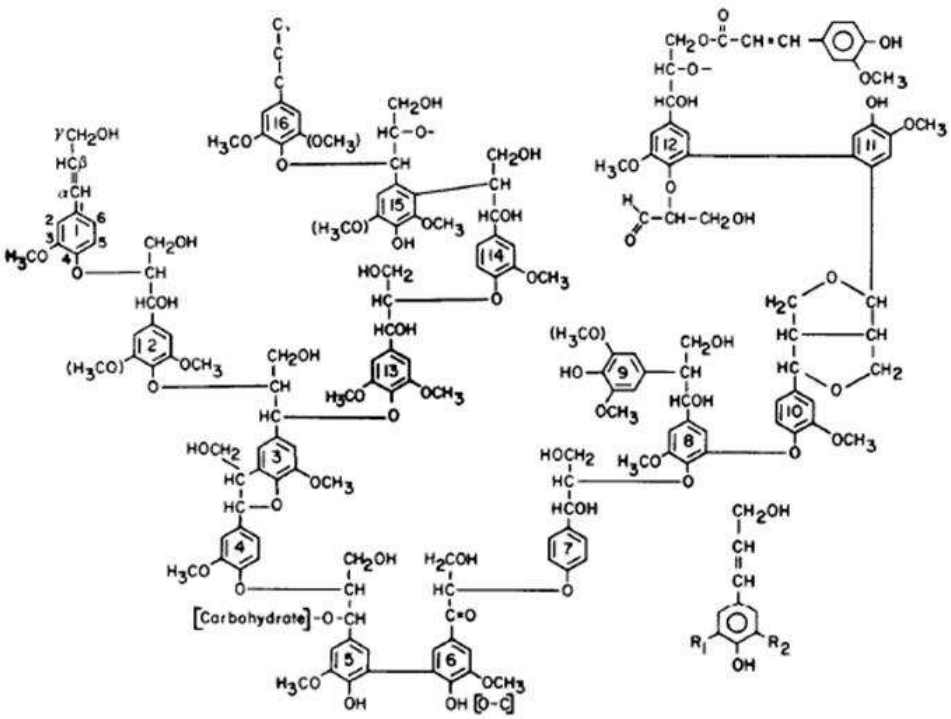
II. Lignin degradation

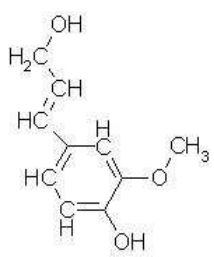
Lignin is the most recalcitrant of all naturally produced organic chemicals. This is consistent with its biological functions, which are to give vascular plants the rigidity they need to stand upright and to protect their structural polysaccharides (cellulose and hemicellulose) from attack by other organisms. When vascular plants die or drop litter, lignified organic carbon is incorporated into the top layer of the soil. This recalcitrant material has to be broken down and recycled by microorganisms to maintain the Earth's carbon cycle (Hammel, 1997)

1. Lignins

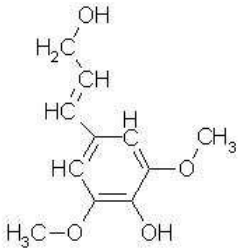
Lignin is an amorphous, three-dimensional polymer. Biosynthetically, lignin arises from free-radical coupling of three phenylpropanoid precursors (Figure I-5) (Kirk and Farell, 1987):

- Coumaryl alcohol,
- Coniferyl alcohol (the guaiacyl units),
- Sinapyl alcohol (the syringyl units).

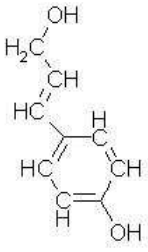




Coniferyl alcohol



Synapil alcohol



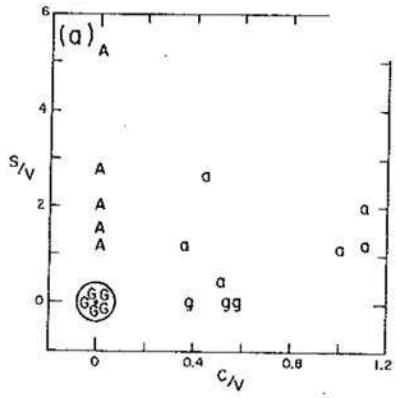
Coumaryl alcohol

Figure I-5: Schematic structural formula for lignin. The structure illustrates major interunit linkages; it is not a quantitatively accurate depiction of the various substructures. The three precursor alcohols are also shown; their polymerisation, following one-electron oxidation, produces lignin (adapted from Kirk and Farell, 1987).

Cupric oxide oxidation (Hedges and Ertel, 1982) is a relatively mild oxidation yielding a suite of single ring phenols (acids, aldehydes and ketones). According to their characteristics, they are grouped to vanillyl compounds (V), syringyl compounds (S) or cinnamyl compounds (C), which comes from lignin constituents, respectively from coniferyl, sinapyl and coumaryl alcohols (Karroum *et al*., 2004). More information about cupric oxide oxidation is given in Part IV, Chapter 3.

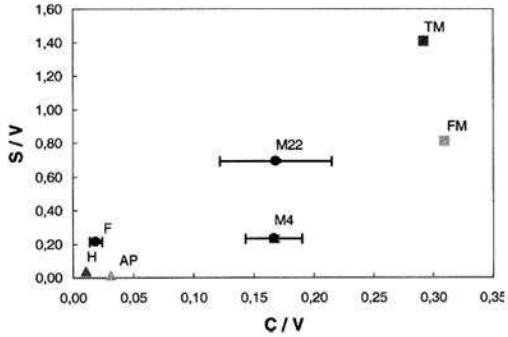
The proportion of each phenylpropanoide moiety in lignin differs among plant species, among plant parts and even within plant cell walls (Sanger, 1996). In the gymnosperms, lignin is composed mainly of guaicyl units (= V compounds) whereas angiosperm lignins contain equal amounts of guaiacyl and syringyl units (= V and S compounds).
The relative contribution of V, S and C units in soils and sediments may reflect the source vegetation. For example, the representation of the S/V *vs* C/V ratios allows us to distinguish between the organic matter of angiosperm and gymnosperm taxons in soils (Hedges and Mann, 1979).

Figure I-6 gives some examples of relationships between S/V and C/V for different plant species and soils.



From Hedges and Mann (1982).

Symbols:
(G) – gymnosperm woods,
(g) – nonwoody gymnosperm tissues,
(A) – angiosperm woods,
(a) – non woody angiosperm tissues,
(n) – non vascular plant tissues.



From Jolivet *et al.*, 2001

Symbols: (F) – A horizon of forest soil, (M4 & M22) – L horizon of cultivated soils aged 4 and 22 years, (H) – forest floor, (AP) – maritime pine leaves, (TM & FM) – maize stems and leaves.

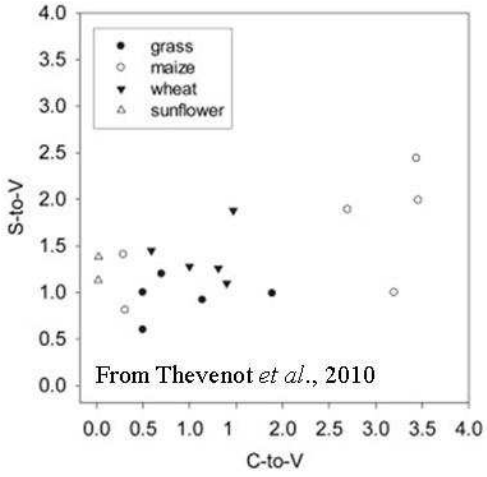


Figure I-6

2. Ligninolysis

The structural features of lignin impose unusual constraints on biodegradation systems responsible for initial attack (Kirk and Farell, 1987):

- Since the lignin is an extremely large and highly branched polymer, ligninolytic mechanisms must be extracellular.
- Since lignin is interconnected by stable ether and carbon-carbon bonds, these mechanisms must be oxidative rather than hydrolytic.
- Since lignin consists of a mixture of stereoirregular units, ligninolytic agents have to be much less specific than typical biological catalysts.
- Finally, the fact that lignin is insoluble in water limits its bioavailability to ligninolytic systems and dictates that ligninolyis is a slow process (Hammel, 1997).

o Enzymes involved in lignin degradation

Lignin degradation is described as an *enzymatic combustion* (Kirk and Farell, 1987). Lignin is degraded with the help of different non-specific oxidoreductases which attack the aromatic moieties. The most important enzymes in this group are presented in Table I-1.
Manganese peroxidase (MnP) is considered to be the most common lignin-modifying peroxidase produced by almost all wood-colonizing basidiomycetes. Its catalytic cycle is presented in point IV of this section.

Table I-1: Enzymes involved in the degradation of lignin and their main reactions (Hatakka, 2001).

Enzyme activity	**Cofactor or substrate "Mediator"**	**Main effect or reaction**
Lignin peroxidase (LiP)	H_2O_2, veratryl alcohol	Aromatic ring oxidized to cation radical
Manganese peroxidase (MnP)	H_2O_2, Mn, organic acid as chelator, thiols, unsatured lipids	Mn(II) oxidized to Mn(III), chelated Mn(III) oxidizes phenolic compounds to phenoxyl radicals; other reactions in the presence of additional compounds.
Laccase (Lacc)	O_2, mediators, e.g., hydroxybenzotriazole or ABTS	Phenols are oxidized to phenoxyl radicals; other reactions in the presence of mediators.
Glyoxal oxidase (GLOX)	Glyoxal, methyl glyoxal	Glyoxal oxidized to glyoxylic acid; H_2O_2 production
Aryl alcohol oxidase (AAO)	Aromatic alcohols (anisyl, veratryl alcohol)	Aromatic alcohols oxidizes to aldehydes; H_2O_2 production
Other H_2O_2 producing enzymes	Many organic compounds	O_2 reduced to H_2O_2

- Microorganisms involved in lignin degradation

The organisms mainly responsible for lignin degradation are aerobic filamentous fungi, and the most rapid degraders in this group are basidiomycetes (Kirk and Farell, 1987). The ability to degrade lignocellulose efficiently is thought to be associated with a mycelium growth habit, which allows the fungus to transport scarce nutrients, e.g. nitrogen and iron, over distance into the nutrient-poor lignocellulosic substrate that constitutes its carbon source.

Brown-rot fungi

Brown-rot fungi comprise a relatively small group of basidiomycetes that decay the cellulose in wood preferentially. They invade the lumens of wood cells, where they secrete enzymes that decompose and remove the polysaccharides, leaving behind a brown, modified lignin residue. Studies show that the lignin undergoes limited aromatic hydroxylation and ring cleavage, but the major effect is demethylation of aromatic groups (Kirk and Farell, 1987). The residual modified lignin they leave behind is an important humus precursor (Hudson, 1986).

White-rot fungi

These are the most abundant degraders of wood in nature (Figure I-7) (Hammel, 1997). The white-rot basidiomycetes degrade lignin more rapidly and extensively than other microbial groups; under optimal conditions, the rates at which they mineralize lignin rival their rates of polysaccharide degradation. Like the brown-rot fungi, they invade the lumen of wood cells, where they secrete enzymes that degrade lignin and other wood components. During its mineralization by white-rot fungi, lignin undergoes a number of oxidative changes, including aromatic ring cleavage. The enzymology and molecular biology of lignin degradation has been mainly studied in *Phanerochaete chrysosporium*, the most widely examined lignin-degrading organisms (Kirk and Cullen, 1998).

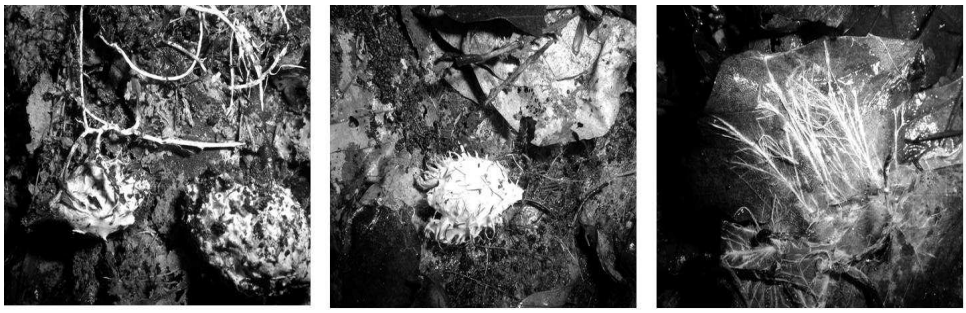


Figure I-7: Wood-rotting basidiomycetous fungi cause white rot in the forest floor of Transinne, Belgium (pictures taken in December 2005).

Soft-rot fungi

Soft-rot fungi (ascomycetes and deuteromycetes) are slower and less aggressive decayers than white and brown-rot fungi (Blanchette, 1991).

Bacteria

Non-filamentous bacteria can usually degrade only the low molecular weight part of lignin as well as the degradation products of lignin. Thus, they may play some role in final mineralization of lignin. Bacterial lignin degradation has been most extensively studied in actinomycetes, particularly *Streptomyces* spp.

o Process of lignin degradation

In most cases, leaf litter decomposition is complex, involving a succession of biodegradative activities that precede attack by lignocelluloseic degraders (Hudson, 1986). The process typically begins with colonization by bacteria and soft-rot fungi that consume the least recalcitrant component present (e.g. sugar, starch and low molecular weight extractives). The cellulose present in non-lignified leaf tissues is then attacked by some of these organisms. Finally, the modified but still lignified litter is colonized by basidiomycetes that degrade it further.

3. Which factors influence ligninolysis?

Nitrogen

In *Phanerochaete chrysosporium*, lignin is degraded only during secondary metabolism (idiophasic), which is triggered by limiting N-nutrients. Lignin degradation by several other species, but not all white-rot fungi, is stimulated by N-limitation (Kirk and Farell, 1987).

Oxygen

Oxygen availability increases the lignin degradation as this process is essentially oxidative (Kirk *et al.*, 1978). Anoxic conditions inhibit ligninolysis, contributing for example, to organic matter accumulation in peat (Gobat *et al*., 1998).

The quality of lignin

In terms of biodegradability by white-rot fungi, vanillyl moieties (V) have a significantly slower rate of decomposition than syringyl moieties (S) (Ander *et al*., 1984).

pH

The pH effect reflects the pH impact on the activity of the fungi in soils; optimal activity registers around pH 5 (Kirk *et al*., 1978).

Manganese

In addition to organic acids, manganese is of course needed for the manganese peroxidase (MnP) activity. Moreover, manganese appears to regulate LiP and MnP production. In low concentrations of Mn(II), extracellular LiP predominates, whereas in the presence of higher concentrations of Mn(II), MnP is dominant (Bonnarme and Jeffries, 1990).

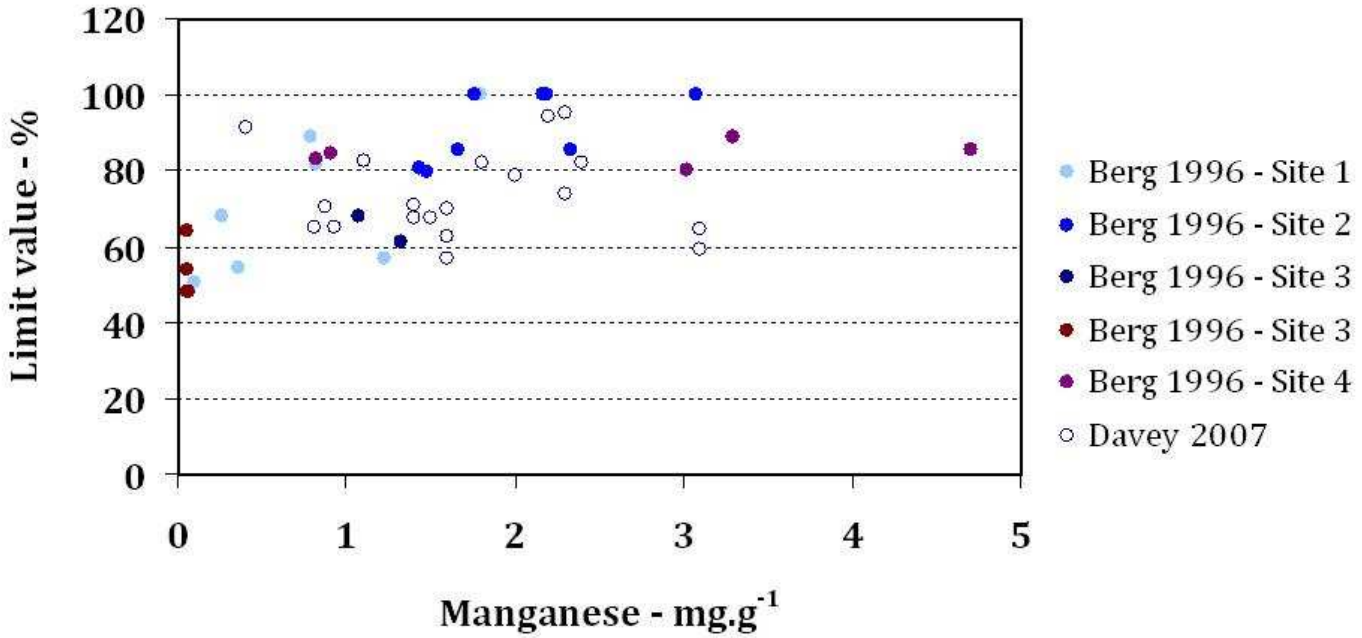


Table I-8: Relationship between limit value (%) of litter and its initial manganese concentration ($mg.g^{-1}$) (the data come from the studies of Berg *et al*. (1996) and of Davey *et al*. (2007*)*

III. Review of papers studying the impact of manganese on litter decomposition

Berg *et al.* (1996) were the first researchers to show a positive correlation between the initial manganese concentration in litter and the level of limit value.

In their study, they analysed 26 litter samples for each of which initial manganese concentration was determined (ranged from 0.050 to 4.71 $mg.g^{-1}$) and limit value was estimated using an asymptotical function (Howard and Howard, 1974). These samples of 13 different tree species (deciduous and coniferous) came from 5 different sites. The data are presented in Figure I-8. In spite of the significance of the observed positive correlation (P = 0.0003), the correlation coefficient (r = 0.44) was not very high.

It is difficult to dissociate this relationship from the effects of site and tree species. By analysing the data of Berg *et al.* (1996), we found that site affected both limit value (P = 0.0004) and initial manganese concentration in litter (P = 0.0012) (Figure I-9). The site would represent the biogeochemical background and/or the pe-pH conditions. The tree species would also impact the manganese concentration in litter. For example, on the same site (site B), litters of different tree species presented contrasting initial manganese concentrations: sugar maple (0.82 $mg.g^{-1}$), white pine (0.91 $mg.g^{-1}$), Aspen (3.02 $mg.g^{-1}$), white oak (3.29 $mg.g^{-1}$) and red oak (4.71 $mg.g^{-1}$).

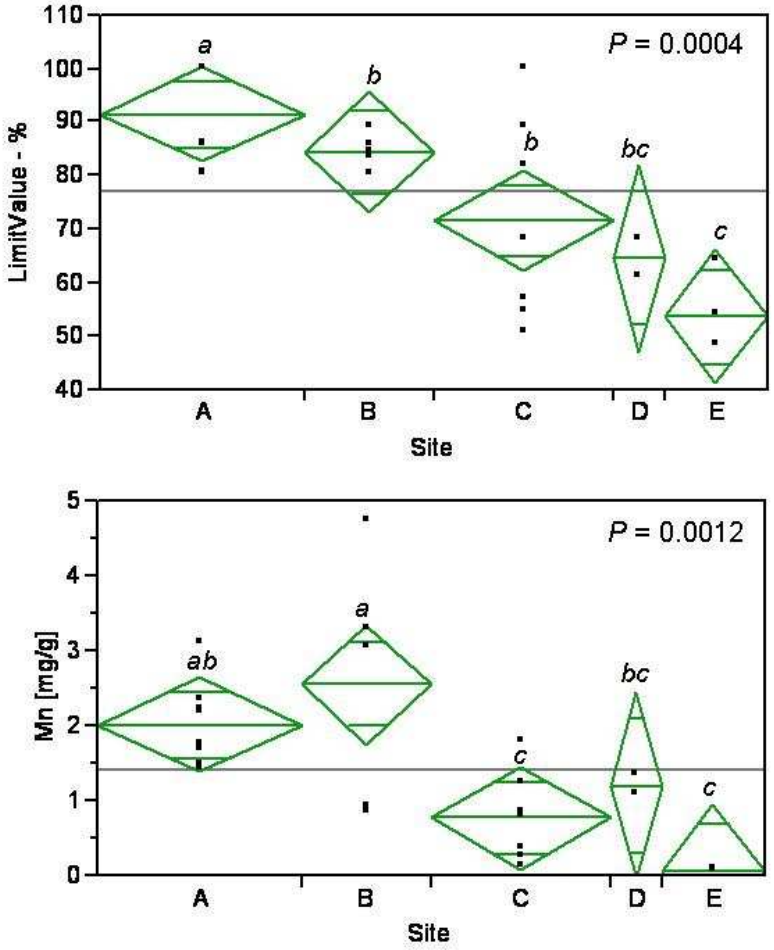


Figure I-9: Box-plots with data of limit value (%) and manganese concentration (mg.g^{-1}) for each site (Berg *et al*., 1996). For each site, means followed by different letters are significantly different from one another at the $P < 0.05$ level according to Fisher's least significant difference test.

Heim and Frey (2004) analysed the data of 10 litters which came from 5 sites ranging from 480 to 1500 m in altitude and including calcareous and acid soils. In their study, they introduced the *decomposition stage* factor by studying litter after one year and after two years of decomposition.

We noted that the range of manganese concentrations studied here is narrower than that studied by Berg *et al.* (1996). They showed a good correlation between the initial manganese concentration in litter and the soil pH for aboveground (R^2 = 0.82) and belowground litter (R^2 = 0.63). Heim and Frey concluded that manganese concentration in fresh litter depended on soil pH rather than on total manganese concentration in the soils.

Moreover, they observed very good correlations between the initial manganese concentration in litter and the mass remaining after one year (r = -0.80, *P* = 0.0049) or two years of decomposition (*r* = -0.83, *P* = 0.0029) (Figure I-10). The slope of linear model of these relations was significantly larger for mass remaining after two years of decomposition than for mass remaining after one year of decomposition (-23.5 *vs* -40.5, *P* < 0.05). Manganese impact would be even more pronounced when the decomposition is advanced.

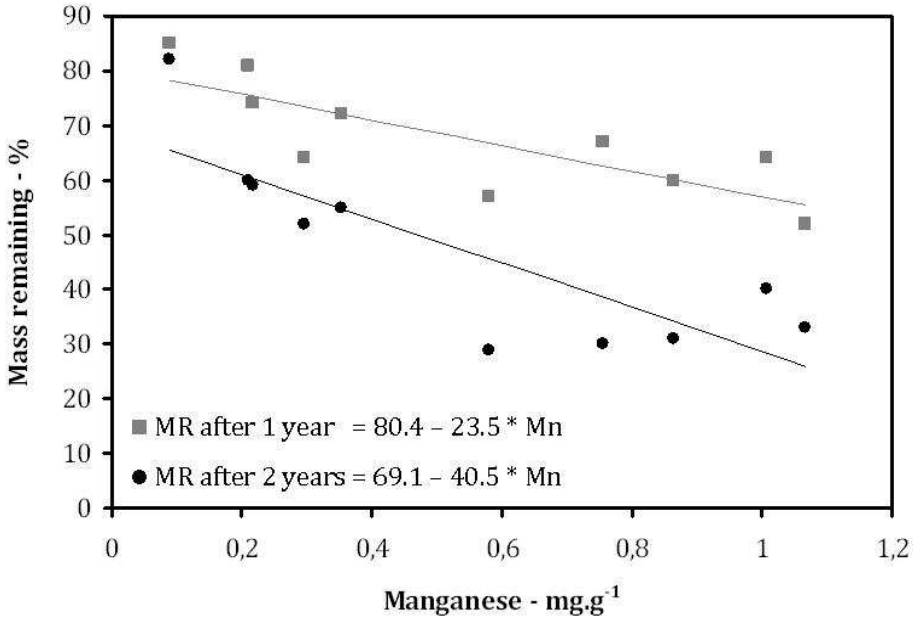


Figure I-10: Remaining mass of litters (%) after one and two years of decomposition as a function of manganese concentration ($mg.g^{-1}$). Data from Davey *et al*. (2007).

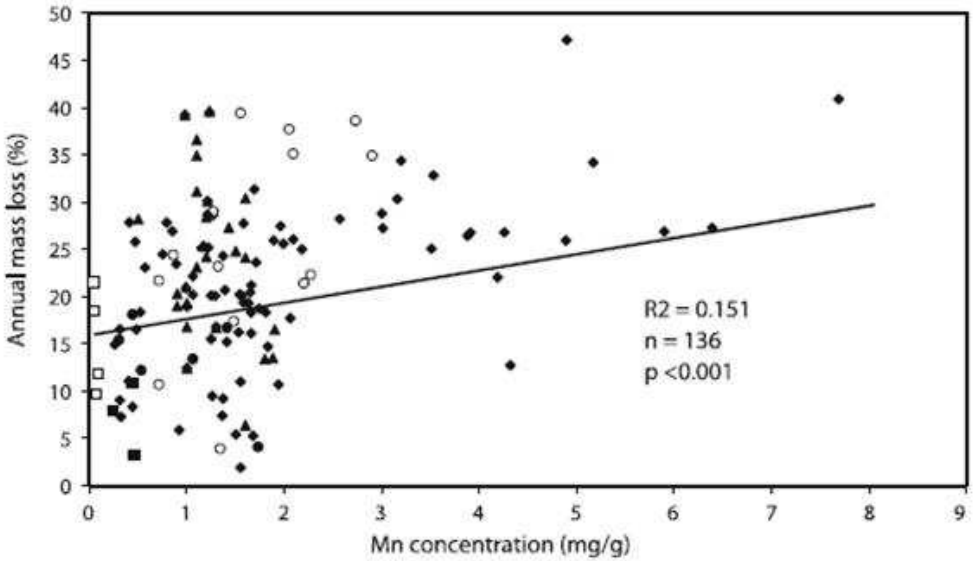
Berg *et al.* (2007) studied the link between initial manganese concentration and the mass remaining in the late stage of decomposition, which was identified as that reached by litter decomposing for more than one year.

Their data set was constituted by 136 samples from 24 different sites including 6 different tree species. The correlation presented in this study (Figure I-11) was significant but had a very low coefficient of determination (R^2 = 0.20). The range of manganese concentration in litter was very broad (0.04 to 7.69 $mg.g^{-1}$) but most of the litters had manganese concentration below 2 $mg.g^{-1}$. Figure I-11 shows that initial manganese concentration seems to be mediated by the tree species.

Berg *et al*. (2007) divided their data set into different groups and showed that:

- When the data were divided by the tree species, the only significant correlation concerned the Norway spruce (R^2 = 0,198, $P < 0.0001$, n = 74).
- When the data were divided by the decomposition stage (according to a lignin concentration of 475-509 mg/g), the relationship between annual litter mass loss and manganese concentration was better (R^2 = 0.46).

The studies of Davey *et al.* (2007) and Berg *et al*. (2010) each considered one tree species in particular, the oak and the pine, respectively.
In the study of Davey *et al.* (2007), the oak (*Quercus robur* L.) leaf litters came from 20 woodland sites ranging from 50 to 330 m in altitude and including acid podzolic soils and underlying lower Palaeozoic rocks.
The correlation between initial manganese concentration in litter and the estimated limit values (Figure I-8) was not significant while the correlation between the initial manganese concentration in litter and the estimated decomposition rate (k) was significant (r = 0.32) (Figure I-12). As the initial manganese concentration is related to initial degradation rates (k), Davey *et al.* (2007) concluded that the degradation of lignin was already important in the early stage of decomposition.



Relationship between annual mass loss in late stages of decomposition of Norway spruce, lodgepole pine, Scots and Aleppo pine, silver birch, grey alder foliage litter and its Mn concentration at the start of each year. Data from 40 sites in Sweden and two sites in northern Libya. (◆) Norway spruce, (▲) Scots pine, (○) lodgepole pine, (□) Aleppo pine (●) silver birch, (■) grey alder

Figure I-11: from Berg *et al.*, 2007.

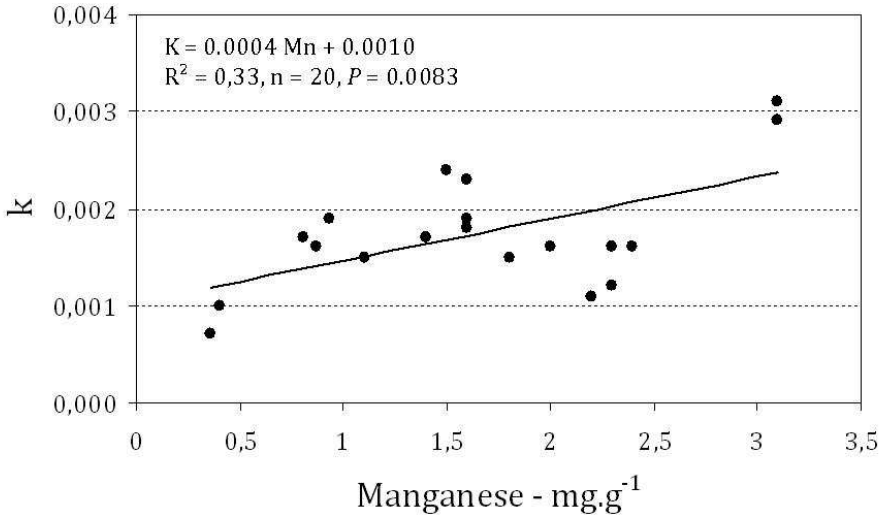


Figure I-12: Linear relationship between decomposition rate (*k*) and initial concentration of manganese in oak leaf litters (from Davey *et al*., 2007).

Berg *et al.* (2010) studied pine needle litters of different genera from 15 different sites. The range of manganese concentration was 0 to 3 $mg.g^{-1}$. They observed no significant effect of manganese concentration when just one site was studied; they therefore concluded that the effect may be related to other factors that vary in concentration.

Figure I-13 gives an outline of these 5 studies and summarizes their main conclusions. Although the causal relationships are not clear and the studies published do not seem to be unanimous about the exact effects of manganese on lignin degradation, it nevertheless seems as though there was a significant relation between manganese concentration and levels of decomposition.
However the relationship is not unequivocal and seems to be dependent on early stage? on late stage? on litter tree species? on critical concentration of manganese in litter?

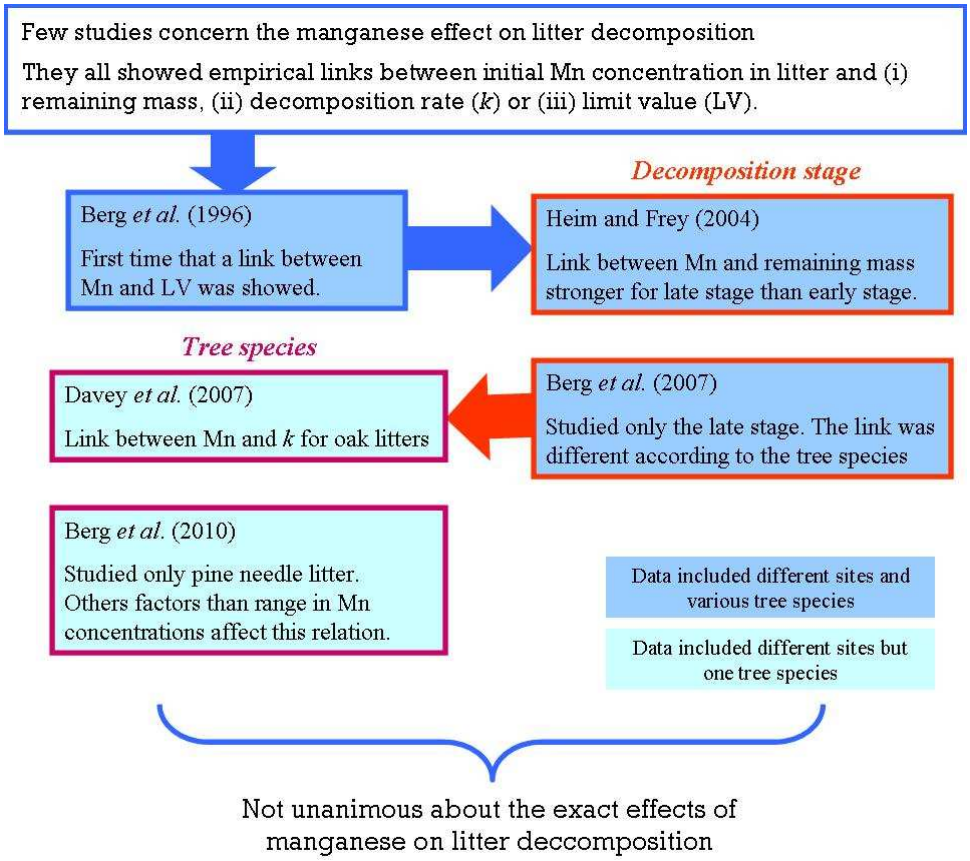


Figure I-13: Summary of the published studies concerning the impact of manganese on litter decomposition.

IV. Several functions of manganese in the organic matter decomposition process

In fact, the organic matter decomposition involves a wide range of processes such as, for example, carbon mineralization, nitrification, ligninolysis and nutrient leaching. This review aims to summarize those processes linked to organic matter decomposition in which manganese may play a part. The processes considered are (i) nitrogen transformations, (ii) microbial activity and (iii) ligninolysis. First, the forms and main functions of manganese are briefly noted.

1. Forms and main functions of manganese

Manganese is the tenth most abundant element in the Earth's crust. This metal does not occur naturally but is combined with other elements in thousands of recognizably different minerals, commonly as a minor constituent.

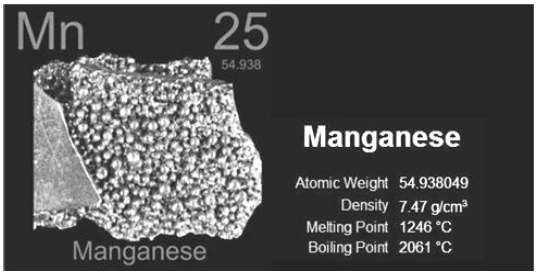


Figure I-14: Manganese is the 25th element of the Mendeleev table

The most obvious features of the chemistry of manganese in simple aqueous solution are the stability of the divalent cation Mn^{2+} in acid solution and the greater stability of MnO_2 in alkaline solution in the presence of O_2. The position of equilibrium between the various forms of occurrence, i.e. the distribution between mobile Mn^{2+} and the insoluble oxides, is largely controlled by drainage, redox conditions, pH (Jarvis, 1984) and characteristics of ligands and surface (Graham *et al.*, 1988).

The pe-pH stability diagram (Figure I-15) identifies the thermodynamically most stable phases within the appropriate regions of pe and pH. The general trends depicted by this figure conform to many aspects of manganese chemistry in soils:

i. Oxide of manganese (III, IV) are generally found in oxidizing environments,
ii. Soluble and exchangeable Mn^{2+} tend to be dominant under strongly acidic conditions,
iii. Mn^{2+} is released when aerobic soils become reduced because of submergence of rapid oxidation of organic materials (Graham *et al.*, 1988).

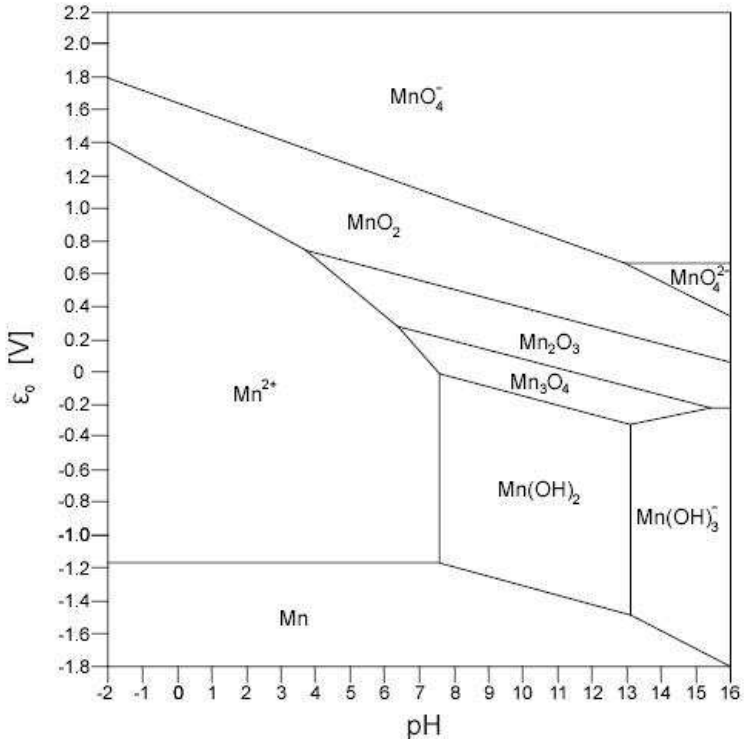


Figure I-15: The pe-pH stability diagram for Mn (Lindsay, 1979)

Given that manganese is a relatively strong electropositive element, it combines with oxygen to form a large range of seven oxidation states which are interchangeable at defined temperature and oxygen pressure (Graham *et al.*, 1988).

The properties of these different oxidation states are visualized on the Frost diagram, plotting the relatively free energy of a species with its oxidation state (Figure I-16). The values to be plotted on the y-axis are obtained by multiplying the number of electrons transferred during an oxidation state change by the standard reduction potential for that change.

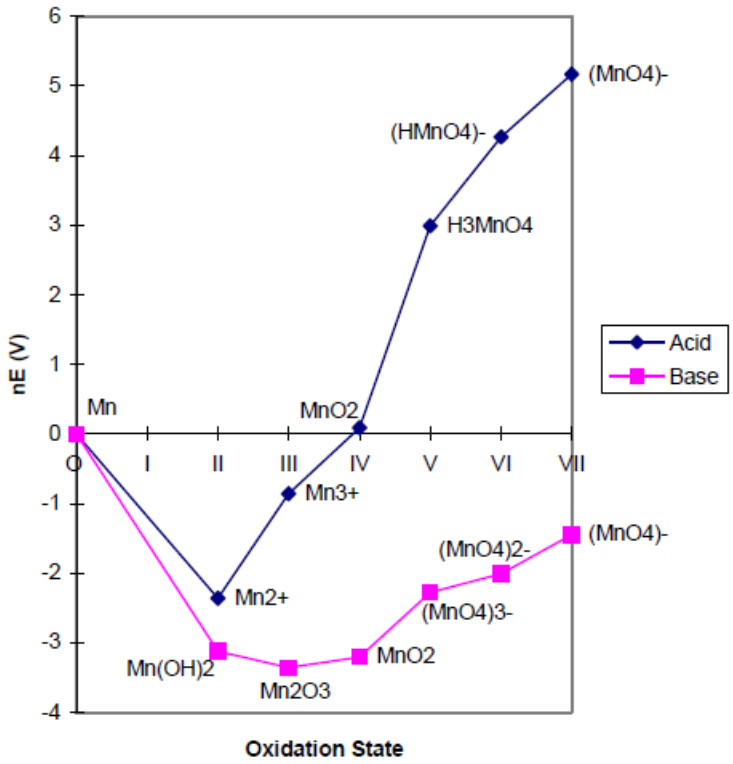


Figure I-16: The Frost diagram for manganese (Graham *et al.*, 1988).

The behaviour of manganese in soils and plants closely reflects certain aspects of its chemistry. The first functional value of manganese is a Lewis acid (compare to Mg and Ca), and the second, in higher oxidation states, is an oxidation catalyst (compare to Fe and Cu). Manganese is involved in many physiological processes within the plant. However, its major functions by far are in oxygen evolution in photosynthesis. Manganese plays at least two roles in this process, being involved both in the water-splitting reaction and in maintaining the structures of the stacking of the chloroplast lamellae (Graham *et al.*, 1988).

2. Involvement of manganese in N-transformations

o The classic nitrogen cycle

Given that nitrogen is an important nutrient in the global biogeochemical system, an understanding of the nitrogen cycle is essential.

The main processes involved in the classic N-cycle are the following:

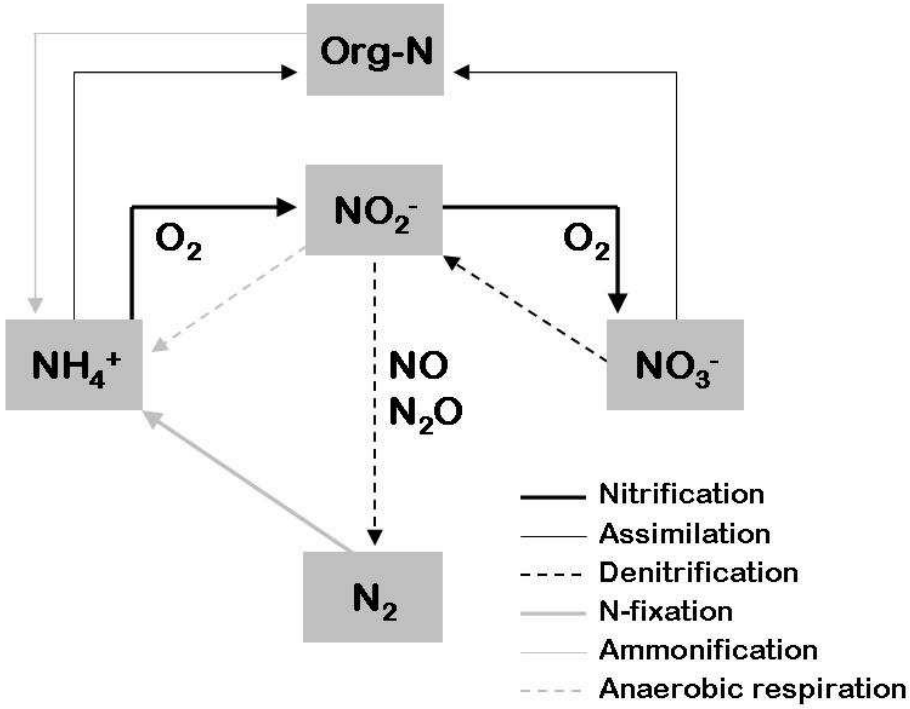


Figure I-17: The classical nitrogen cycle (adapted from Newton, 2006).

- NO_2^- and NO_3^- are formed through the process of oxidizing NH_4^+ (**nitrification**), which classically requires oxygen (Hulth *et al.*, 1999). Different organisms are involved in the oxidation of NH_4^+ to NO_2^- (e.g. *Nitrosomas, Nitrospira* or *Nitrosococcum* bacteria) and in the oxidation of NO_2^- to NO_3^- (e.g. *Nitrobacter, Nitrospira* or *Nitrococcus* bacteria).

- NH_4^+ and NO_3^- are also integrated into organic matter through **assimilation** (Aber, 1992). Growth by organisms requires N (NH_4^+ and NO_3^-) which is incorporated in biomass (Piersinsky *et al.*, 2000).

- **Denitrification** is the pathway through the electron transport chain that reduces NO_3^- to N_2. Denitrification requires the NO_3^- to reduce back to NO_2^- before following the classic denitrification chain.

- **Nitrogen fixation** is not the only pathway for NH_4^+ production. **Ammonification** is the pathway of NH_4^+ release into solution from organic matter degradation. Also, reduction of NO_2^- to NH_4^+ is assumed as **anaerobic respiration** of NO_2^- (Piersinsky *et al.*, 2000).

o An alternative pathway for denitrification and nitrification

The sedimentary nitrogen cycle coupled to the manganese cycle gives an alternative pathway for denitrification and nitrification processes (Luther *et al.*, 1997). These reactions are thermodynamically favourable under environmentally realistic conditions (Luther *et al.*, 1997).
A schematic diagram of these reactions is given in Figure I-18.

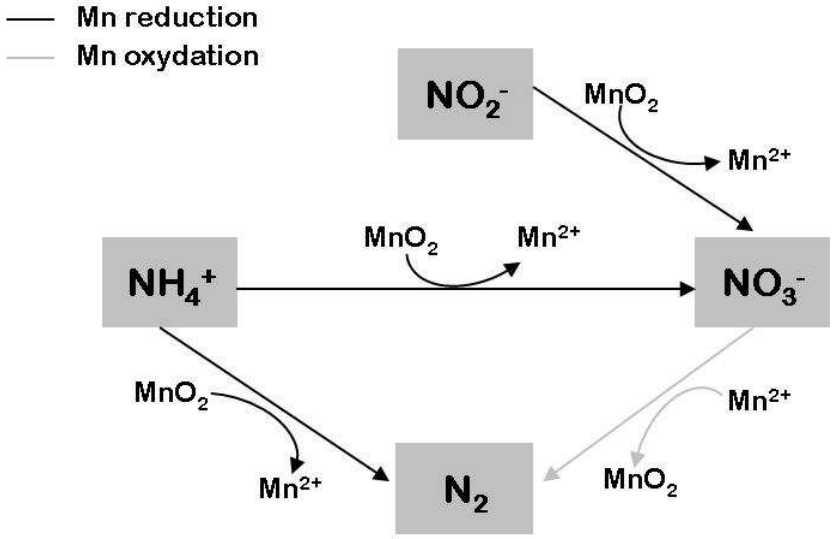


Figure I-18: Diagram of Mn reactions involved in N-cycling (Newton, 2006).

➢ Denitrification

$NH_4^+ + 3/2\ MnO_2 + 2\ H^+ \rightarrow N_2 + 3/2\ Mn^{2+} + 3\ H_2O$ **Eq. I-2**

$NO_3^- + 5/2\ Mn^{2+} + 2\ H_2O \rightarrow 1/2\ N_2 + 5/2\ MnO_2 + 4\ H^+$ **Eq. I-3**

➢ Nitrification

$4\ MnO_2 + NH_4^+ + 6\ H^+ \rightarrow 4\ Mn^{2+} + NO_3^- + 5\ H_2O$ **Eq. I-4**

$MnO_2 + NO_2^- + 2\ H^+ \rightarrow Mn^{2+} + NO_3^- + H_2O$ **Eq. I-5**

Firstly, the non microbial conversion of NO_2^- to NO_3^- by manganese oxides (Eq. I-5) may explain the lack of accumulation of NO_2^- in soils in spite of low numbers of *Nitrobacter* bacteria and conditions unfavourable to their growth (Bartlett, 1981). The Bartlett's experiment (1981) showed that nitrate formation in soils equilibrated with nitrite at 0.5°C was directly related to the soil level of reactive manganese oxides. It was demonstrated that synthetic manganese oxides can oxidize nitrite to nitrate and become reduced in the process. Nitrite oxidation, nitrate formation and MnO_2 reduction were stoechiometrically related reactions in the presence or absence of atmospheric O_2.

Secondly, the anoxic oxidation of ammonia by manganese oxides (Eq. I-2) is a newly recognised pathway for the production of N_2 in sedimentary environments, which potentially contributes to a significant loss of N from the world's oceans. This important link in the global cycle is traditionally assumed to take place via the coupled bacterial nitrification-denitrification processes (Bartlett *et al.*, 2007). Luther *et al.* (1997) provided field and laboratory evidence that N_2 can also be produced by the oxidation of NH_3 and org-N with MnO_2 in air.

Lastly, the interest of Mn-mediated nitrification is that nitrification may occur anaerobically. In anoxic sediments, the potential for manganese oxides to serve as oxidant for nitrification may be considerable. Therefore, the Mn-mediated nitrification was preferentially studied through marine sediment incubation (Bartlett *et al.*, 2008; Hulth *et al.*, 1999; Luther *et al.*, 1997).

These studies concluded that the introduction of manganese oxides from oxidizing surface sediment into the underlying anoxic region during biogenic or physical mixing results in anoxic nitrification and net production of NO_2^- and NO_3^- (Hulth *et al*., 1999). Anoxic nitrification may not only be dependent on total manganese concentration in the solid phase (Hulth *et al*., 1999) but also on manganese dynamics (Bartlett *et al*., 2008).

3. Involvement of manganese in microbial activity

The ability of soil microorganisms to oxidize and reduce manganese in axenic culture has been recognized for many years; however, specific taxonomic groups of *manganese bacteria*, analogous to *iron bacteria* or *nitrifying bacteria*, have never been identified (Graham *et al*., 1988).
Mn-reducing microorganisms isolated from soil include an assortment of bacteria and fungi that produce H_2S, H_2O_2, organic acids or other metabolic end products that can reduce manganese oxides abiotically.

In addition, some bacteria appear to have enzymatic mechanisms for reducing manganese, and some even appear to be capable of anaerobic *manganese respiration* (Ehrlich, 1987; Ghiorse, 1988).

Thermodynamic calculations (Ehrlich, 1976) show that at neutral pH, the oxidation of Mn^{2+} can supply free energy to support chemolithotrophic growth:

$$Mn^{2+} + 1/2\ O_2 + H_2O \rightarrow MnO_2 + 2\ H^+ \qquad \textbf{Eq. I-6}$$

Delta G_0 = - 16.3 kcal/mol

Among the reasons that have been proposed for the evolutionary selection of this process are protection for damage by toxic metals and oxygen species, protection from ultraviolet light and strengthening of the bacterial sheath or capsule (Ghiorse, 1984).

Sunda and Kieber (1994) reported that manganese oxides lyse complex humic substances, which in general cannot be used by microorganisms directly to form low-molecular-weight organic compounds (e.g. pyruvate) that can be used as substrate for microbial growth. Mn-oxidizing bacteria may thus be able to use the carbon pool in humic substances, which represent one of the largest organic reservoirs in natural waters, sediments and soils.

In their laboratory experiment, Miltner and Zech (1998) showed that birnessite ((Na, Ca) Mn_7O_{14} 2.8 H_2O) promoted organic matter decomposition due to its oxidative effect. They concluded that, as long as manganese oxides are present, there will be a continuous supply of easily degradable substrates for microorganisms and therefore an enhanced decomposition of humic substances.

Manganese toxicity?

Manganese is an essential trace element for all living organisms, but at high concentrations it is toxic, even for microorganisms. The basis for the toxicity is not understood, and is an area that needs more study (Nealson *et al*. 1988).

For example, as an essential trace element, manganese is required at a concentration of 2 – 10 μM for optimal yeast growth and inhibited it at concentrations of 0.5 mM (Blackwell *et al*., 1998). Another experiment showed that when Mn(II) is added to natural lake water or seawater, even at concentrations that might be considered low (10-20 μM), the ability of the microbes in the water to take up and oxidize Mn(II) is markedly inhibited (Nealson *et al.*, 1988). Like many other examples of Mn(II) toxicity, the mechanisms of this inhibition are not understood.

4. Involvement of manganese in ligninolysis

As previously presented in point II of this section, lignin degradation may be considered as an *enzymatic combustion* (Kirk and Farell, 1987). The main extracellular enzymes involved in lignin degradation are lignin peroxidase (LiP), manganese peroxidase (MnP), laccase, versatile peroxidase and H_2O_2 producing oxidase. Studies with different white-rot fungi have shown that MnP, discovered by Kuwahara *et al*. in 1984, appears to be more common than LiP (Orth *et al.*, 1993), and during recent years the characteristics and role of MnP have been extensively studied.

o The catalytic cycle of MnP needs Mn^{2+}

The catalytic cycle of the MnP is schematized in Figure I-19. MnPs complete the conventional peroxidase cycle but preferentially use Mn^{2+}, which is ubiquitous in all lignocellulose and soil, as the substrate. Compounds I of MnP can oxidize phenolic substrates or Mn(II), but MnP compound II is apparently only reduced by Mn(II).

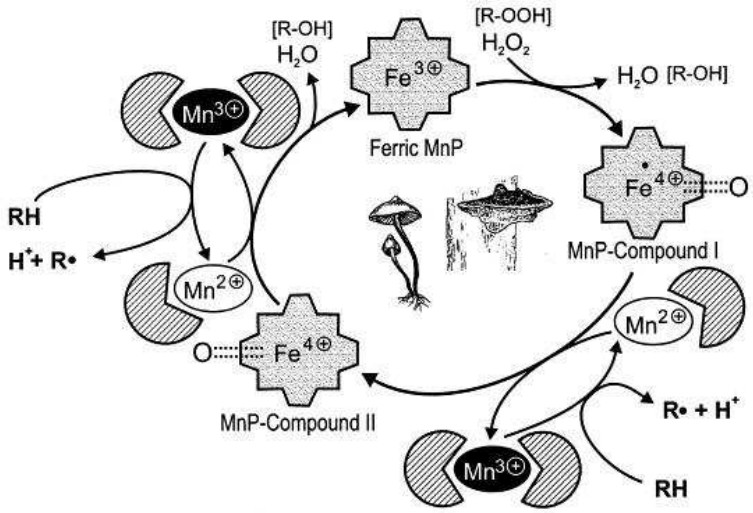


Figure I-19: The catalytic cycle of MnP (Hofrichter, 2002).

The reactions involved in the MnP catalytic cycle are the following:

$MnP(Fe^{3+})P + H_2O_2 \rightarrow MnP - I(Fe^{4+}\text{-}O)P^{*} + H_2O$ **Eq. I-7**

$MnP - I(Fe^{4+}\text{-}O)P^{*} + Mn^{2+} \rightarrow MnP - II(Fe^{4+}\text{-}O)P + Mn^{3+}$ **Eq. I-8**

$MnP - II(Fe^{4+}\text{-}O)P + Mn^{2+} \rightarrow MnP(Fe^{3+})P + Mn^{3+} + H_2O$ **Eq. I-9**

In this scheme, P is porphirin, MnP compound I (MnP-I) carries both oxidizing equivalents of H_2O_2, one of oxyferryl center and one as a porphyrin pi cation radical (P^{*}). MnP compound II (MnP-II) carries only one oxidizing equivalent.

- Stabilization of the resulting Mn^{3+} by chelation

MnP oxidizes Mn^{2+} to Mn^{3+}, which is stabilized by organic acids such as oxalate, malate, lactate and malonate chelating the oxidized manganese ions (Kirk and Cullen, 1998). The resulting Mn(III) chelates are small, diffusible oxidants that can act at a distance from the MnP active site (Hammel, 1997). This stabilization of Mn^{3+} appears to be a key requirement for the MnP ligninolytic activity given that Mn^{3+} chelated by suitable organic acids has been shown to be a powerful oxidative complex able to attack various organic molecules (Forrester *et al.*, 1998).

An organic acid chelator in the MnP system:

- Facilitates the dissociation of Mn^{3+} from the enzyme,
- Stabilizes Mn^{3+} with a high redox potential,
- Has a relatively low binding constant for Mn^{2+}, allowing the enzyme access to free Mn^{2+} (Wariischi *et al.*, 1992).

Part II

Impact of tree species on litter decomposition: focus on manganese concentration

This part uses the data of Trum F., Titeux H., Ranger J. and Delvaux B., 2011. Influence of tree species on carbon and nitrogen transformation patterns in forest floor profiles. Annual Forest Science DOI 10.1007/s13595-011-0080-4.

In this part, we focus on manganese concentration in forest floor in the scope of our study entitled "*Influence of tree species on carbon and nitrogen transformation patterns in forest floor profiles*" (Trum *et al*., 2011b). First, we summarize the study such as it was published (the original paper is presented in appendix 1, page 156). Then, we highlight some points concerning the relationships between manganese concentration in forest floor horizons and (i) carbon mineralization and (ii) nitrification degree.

I. Summary of the study

The impact of tree species on soil is ruled by strong soil-plant feedbacks and interactions with climate. Using forest stands involving different tree species in homogeneous soil and climate conditions is thus a prerequisite to understand the specific effect of tree species on soil properties and processes.

Here, we studied the impact of tree species on the forest floor properties in 27-year-old monospecific stands of oak, beech, spruce and Douglas-fir, grown under homogeneous pedoclimatic conditions (submountain Atlantic climate and acid brown soil) in the common garden of Breuil (INRA experimental site, Morvans, France).

In December 2005, forest floors were sampled by collecting each horizon separately (Oln, Olv, Of and O*). We studied the chemical composition of their leachates (DOC, DON, NH_4^+ and NO_3^-) under controlled temperature (4°C) and humidity, and we monitored the release of CO_2.

In spite of similar litterfall C-inputs (1.3–1.8 Mg ha^{-1} yr^{-1}), C-stocks strongly differed between coniferous (16-21 Mg ha^{-1}) and broadleaved (7-9 Mg ha^{-1}) forest floors. The large C-mineralization under oak (196 C-µg C_{total}-g^{-1} d^{-1}) relatively to other stands (125-147 C-µg C_{total}-g^{-1} d^{-1}) and the large dissolved organic carbon release under broadleaved forest floors may explain this difference. Spruce forest floor exhibited the lowest net nitrification and the largest contribution of organic anions to the anionic charge of aqueous leachate (62.5 % ± 1.7). Conversely, Douglas-fir forest floor exhibited the largest relative nitrification and the largest contribution of nitrate to the anionic charge (70.8 % ± 0.7).

We conclude that tree species induces a specific signature in terms of proton donors at the forest floor level (organic *vs* nitric acid), which involves potential impacts on pedogenetic processes in mineral horizon beneath.

II. Tree species have different ability to recycle manganese.

As already observed in the data of Berg *et al.* (1996) (Part I, point III), our data confirm that initial manganese concentrations in litter depend on the ability of tree species to recycle manganese. With a similar biogeochemical background, the initial manganese concentrations ($mg.g^{-1}$) observed in litterfall of tree species were contrasted: spruce, 1.7 ± 0.1 < beech, 2.0 ± 0.1 < oak, 2.3 ± 0.1 < Douglas-fir, 2.7 ± 0.1 (P < 0.0001, n=3). These manganese concentrations in litterfall were in the opposite order of the observed pH in the soil (spruce > beech > oak > Douglas-fir) (Figure II-1). A decrease in pH favours the mobility of manganese under Mn^{2+} form (Lindsay, 1979).

The physiological status of trees controls the manganese acquisition more than the actual available manganese resources in the soil (Albert and Pescoller-Tiefenthaler, 1992).

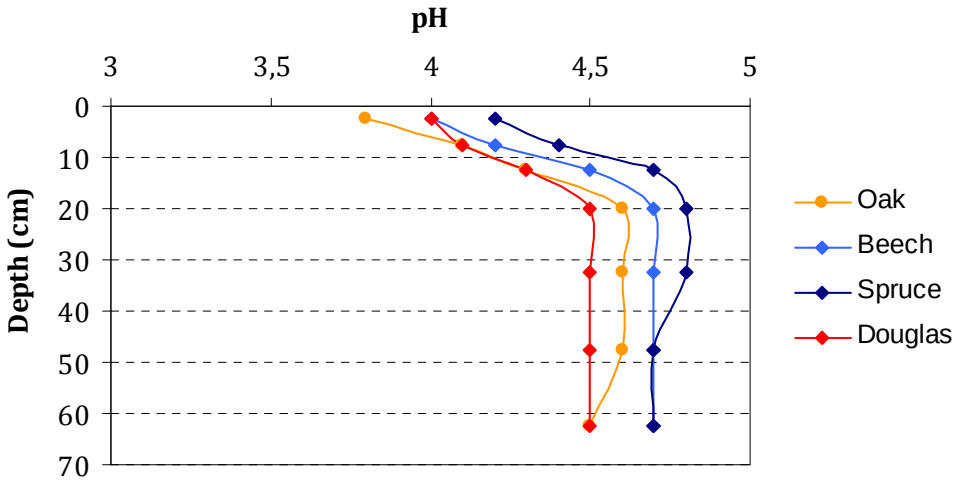


Figure II-1: pH of soils under stands of different tree species (from Ranger *et al.*, 2004).

III. A simple estimator of relative nitrification

For each forest floor horizons, net nitrification (N-µg N_{total}-g^{-1} d^{-1}), relative nitrification (%) and nitrification degree (%) were computed:

- Net nitrification (N-µg N_{total}-g^{-1} d^{-1}) was defined by the ratio between (1) net NO_3^- production, which is the difference between final and initial NO_3^- concentrations in the KCl extracts, and (2) initial amount of N in the sample.
- Relative nitrification (%) is defined by the ratio between (1) final NO_3^- in the KCl and water extracts and (2) final mineral N (NH_4^+ and NO_3^-) in the KCl and water extracts. It is an alternative measure of nitrification considering NO_3^- production in relationship to NH_4^+-supplying capacity (Robertson, 1982).
- The nitrification degree is the ratio between final NO_3^- and final inorganic N (NH_4^+ and NO_3^-) in the water extracts (Ellenberg, 1977) and characterizes the extent which nitrate and ammonium are delivered by the soil.

Analysing the data whatever the forest floor horizon (Oln, Olv, Of, O*) and whatever the forest tree species (oak, beech, spruce and Douglas-fir), we observed a high significant correlation coefficient (0.95) between the relative nitrification (%) and the nitrification degree (%) (Table II-1). According to the definition of these two ratios, the main difference between them concerns the quantity of ammonium on the exchangeable complex.

r (n=16)	**(1)**	**(2)**	**(3)**
(1)		0,60 *P=0.0131*	0,60 *P =0,0136*
(2)	0,60 *P=0.0131*		**0,95** *P<0,0001*
(3)	0,60 *P=0,0136*	**0,95** *P<0,0001*	

Table II-1: Pearson's correlation coefficient (r) between (1) Net-nitrification [N-µg.N_{total}.g^{-1} d^{-1}], (2) Relative nitrification [%] and (3) Nitrification degree [%]. The p-values (*P*) test the hypothesis of no correlation against the alternative that there is a non-zero correlation.

With a simpler experimental procedure, meaning that only water extractions are needed and no more KCl extractions, we obtained a good estimation of relative nitrification values by the nitrification degree values (Figure II-2). The nitrification degree overestimates (double) the relative nitrification. Later in this thesis, we used the values of nitrification degree to compare the impact of manganese concentration on the NO_3^- production in relation to NH_4^+-supplying capacity

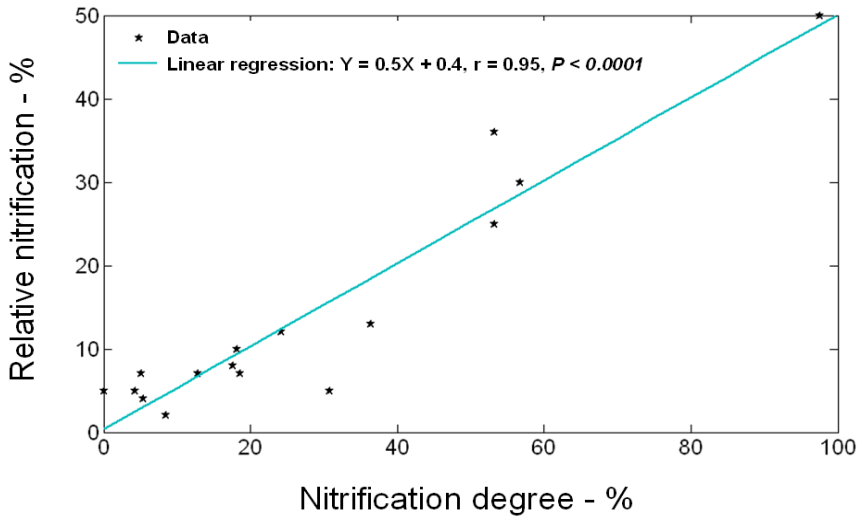


Figure II-2: Linear link between the values of nitrification degree (X-axis) and the values of relative nitrification (Y-axis).

IV. Correlations with manganese concentration

As in the studies presented in Part I, point III (Review of papers studying impact of manganese on litter decomposition), the data analysis shows empirical relationships between manganese concentration and processes linked to organic matter decomposition (Figure II-3):

- A significant positive link between manganese concentration in the incubated forest floor organic materials and their carbon mineralization (CO_2 release) (r = 0.61, *P* = 0.0153). We hypothesise that this link is due to the positive influence of manganese on ligninolysis.

- A significant negative link between manganese concentration in the incubated forest floor organic materials and their nitrification degree (r=-0.68, *P* = 0.0050). We hypothesise that this link is due to the acidifying effect of nitrification contributing to the manganese mobility under Mn^{2+} form.

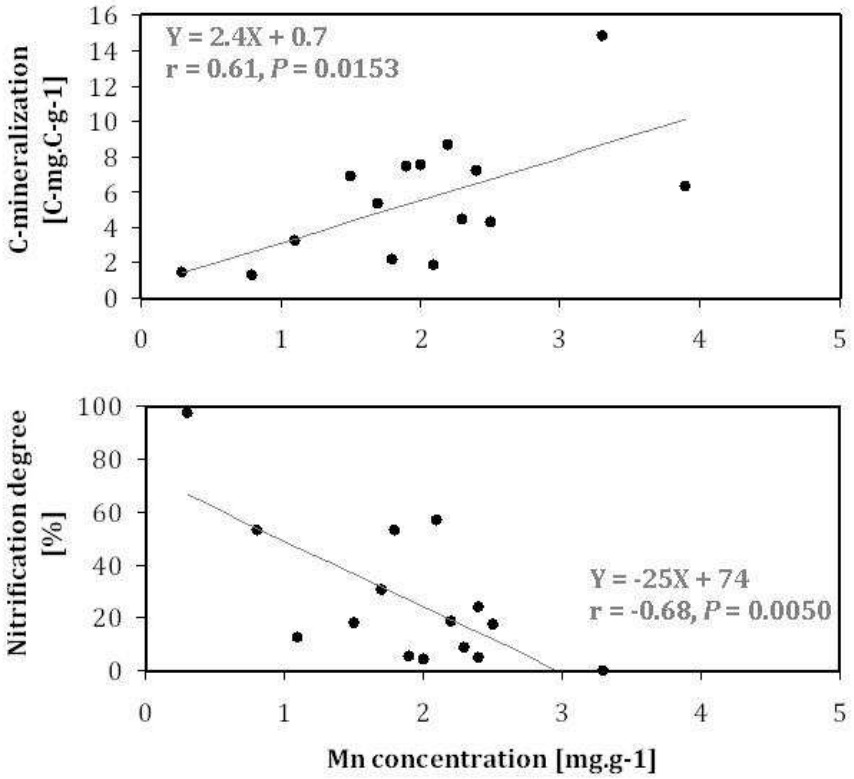


Figure II-3: Linear link between the values of manganese concentration (X-axis) and the values of (i) C-mineralization and (ii) nitrification degree (Y-axis).

- - - - - PHOTOBOARD I - - - - -

Experimental site of Breuil (France)

Established in the Morvan mountains, this site is a common garden set up in 1976 especially to compare biogeochemical cycles between species.



Forest floor horizons sampling in December 2003

- - - - - PHOTOBOARD I - - - - -

Successive horizons of the forest floor (native forest)



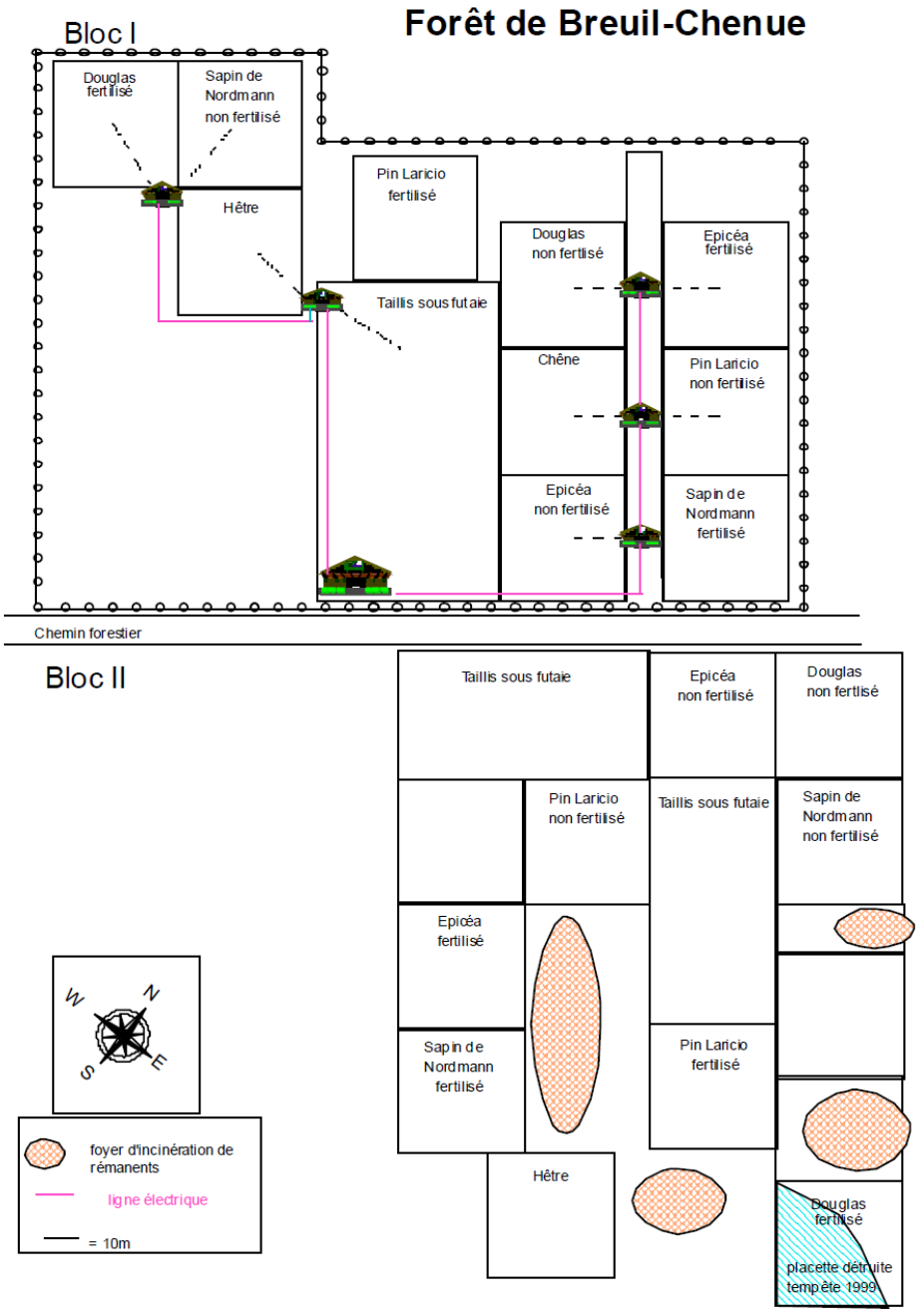












Source: Ranger J. and Gelhaye D., 2006. Présentation du site expérimental de la forêt de Breuil-Chenue. INRA, centre de Nancy (Champenoux).

Part III

Effect of manganese addition on carbon release from forest floor horizons

This part is a slightly modified version from Trum F., Titeux H., Cornélis J-T. and Delvaux B., 2011. Effect of manganese on carbon release from forest floor horizons. Can. J. For. Res. 41: 643-648.

Abstract

Lignin concentration in organic residues largely controls their decomposition. Manganese ion (Mn^{2+}) may well play a key role in ligninolysis because it is a cofactor of manganese peroxidase, an enzyme of the lignin-degrading system. This study aims to investigate the effects of Mn^{2+} addition on forest floor horizon decomposition during laboratory incubation. Therefore, we sampled two distinct forest floors from European beech (*Fagus sylvatica* L.) stands: a mor and a moder. Lignin and Mn concentrations in forest floor upper layer were significantly larger in moder than in mor. Three horizons from each forest floor were separately incubated with or without Mn^{2+} addition (250 mg Mn . kg $_{\text{dry matter}}^{-1}$) and the release of both CO_2 and dissolved organic carbon (DOC) was measured. The DOC release was not impacted by the Mn^{2+} addition, while an increase in CO_2 release from specific horizons was observed. Our data suggest that the impact of the Mn^{2+} addition depends on (i) the forest floor type and on (ii) the organic matter decomposition stage.

Résumé

La teneur en lignine des résidus organiques exerce un contrôle majeur sur leur décomposition. Le manganèse (ion Mn^{2+}) jouerait un rôle clé dans la ligninolyse car c'est un cofacteur de la manganèse peroxidase, l'enzyme principale du système de dégradation de la lignine. Cette étude de laboratoire a pour objectif d'évaluer les effets de l'addition de Mn^{2+} sur la décomposition d'horizons de litière forestière. A cet effet, nous avons échantillonné deux litières de peuplements de hêtre (*Fagus sylvatica* L.): un mor et un moder. Les teneurs en lignine et en Mn sont significativement plus élevées dans l'horizon supérieur du moder que dans celui du mor.

Pour chacune des deux litières, trois horizons ont été incubés séparément avec ou sans addition de Mn^{2+} (250 mg Mn . kg $_{\text{matière sèche}}^{-1}$) et les libérations de CO_2 et de carbone organique dissous (DOC) ont été mesurées. La libération de DOC n'a pas été influencée par l'addition de Mn^{2+} alors que nous avons observé une augmentation de la libération de CO_2 pour certains horizons. Nos résultats suggèrent que l'impact de l'addition de Mn^{2+} dépend (i) du type de litière ainsi que (ii) du stade de dégradation de la matière organique.

I. Introduction

Forest floor decomposition is a key process in the global C cycle on the Earth's surface, controlling the rate of C released in the atmosphere (Heim and Frey, 2004) and stabilized in soil (Lal, 2005), and as such, strongly impacts the global nutrient cycle. Climate and litter quality mainly control the rate of forest floor decomposition. Lignin concentration is usually taken as an indicator of litter quality (Melillo *et al.*, 1982). Indeed, lignin degradation is a very slow process and generally represents the rate-limiting step of organic matter decomposition (Crawford, 1981). Lignin degradation is controlled by various factors such as N concentration (Keyser *et al.*, 1978), medium pH and oxygen concentration (Crawford, 1981).

More recently, several studies (Berg *et al.*, 2007; Davey *et al.*, 2007; Heim and Frey, 2004; Virzo De Santo *et al.*, 2009) highlighted a positive correlation between the rate of forest floor decomposition and the Mn concentration in litterfall given the positive influence of Mn on ligninolysis. Manganese peroxidase, an enzyme of the lignin degrading-system secreted by white-rot fungi, oxidizes Mn^{2+} to highly reactive Mn^{3+}, which penetrates the lignin structure and oxidizes phenolic units (Hammel, 1997; Hofrichter, 2002; Perez and Jeffries, 1992). Perez and Jeffries (1992) also suggested that Mn promoted lignin degradation by stabilizing the lignin-degrading enzymes and by enhancing their production. However, the influence of Mn on forest floor decomposition has not been directly measured so far.

Our study therefore assesses the effects of Mn^{2+} addition on C release in two forest floor types and at various organic matter decomposition stages. For this purpose, samples from three forest floor horizons (Olv, Of2, and Oh) of two distinct forest floors (mor and moder) were incubated after irrigation with either demineralised water or 1.5mmoll^{-1} $MnSO_4$ solution. We determined CO_2 release by respirometry and dissolved organic carbon (DOC) release by leaching.

Table III-1: Major properties of the two studied sites (data from Titeux, 2005).

a. Mor site soil (Soignes)

Horizon	Depth	pH H_2O	Sand	Silt	Clay	C	TRB*	CaO	MgO	Na_2O	K_2O	MnO
	cm			%		% of DM	$cmol_c\ kg^{-1}$			% of DM		
E1	0-12	4.1	80	16	4	1.4	20	0.02	0.03	0.12	0.66	0.01
E2	13-28	4.3	83	12	5	0.4	21	0.03	0.03	0.11	0.68	0.01
Bhs	28-35	3.9	80	9	11	1.3	40	0.04	0.24	0.11	1.11	0.01
Bs1	35-43	4.2	82	8	10	0.7	39	0.02	0.22	0.10	1.14	0.01
Bs2	43-52	4.5	83	8	9	0.4	39	0.03	0.22	0.10	1.11	0.01
2Bg	52-110	4.4	79	4	17	0.2	59	0.01	0.51	0.08	1.47	0.00

b. Moder site soil (Transinne)

Horizon	Depth	pH H_2O	Sand	Silt	Clay	C	TRB	CaO	MgO	Na_2O	K_2O	MnO
	cm			%		% of DM	$cmol_c\ kg^{-1}$			% of DM		
Ah	0-2.5	3.9	21	49	30	19.6	95	0.11	0.34	0.49	1.38	0.00
BC1	2.5-5	4.2	19	58	23	10.7	100	0.10	0.59	0.40	1.97	0.05
BC2	5-10	4.4	17	55	28	6.0	100	0.08	0.66	0.37	1.97	0.09
BC3	10-16	4.4	17	58	25	4.4	106	0.09	0.72	0.44	2.03	0.01
BC4	16-29	4.6	17	60	23	2.7	113	0.09	0.82	0.45	2.17	0.01
Bw1	29-39	4.6	16	62	22	1.4	125	0.09	1.00	0.48	2.36	0.01
Bw2	39-60	4.5	20	55	25	0.4	139	0.10	1.21	0.55	2.48	0.01
BC1	60-80	4.5	27	59	14	0.2	172	0.05	1.39	1.19	2.71	0.01

* The total reserve in bases (TRB) is the sum of total Ca, Mg, K and Na concentrations.

II. Materials and methods

1. Study sites

The European beech (*Fagus sylvatica* L.) is a widespread tree species with broad ecological amplitude. The decomposition of its litter will be more or less rapid in function of the pedoclimatic conditions.

This study was carried out on two distinct forest floors of European beech stands: a mor and a moder. By definition, a moder forest floor exhibits better decomposition dynamics than a mor forest floor (Brêthes *et al*., 1995). The mor was sampled in the Forêt de Soignes near Brussels (Belgium) (50°48'24"N, 4°28'04"E) where the climate is humid temperate, with 835 mm mean annual rainfall and 9.4°C mean annual temperature. The beech stand developed on a Podzol (IUSS, 2006) derived from tertiary sandy sediments. The moder was sampled in Transinne in the Belgian Ardennes (50°11'31"N, 4°59'03"E). This site has a mean annual rainfall of 1034 mm and a mean annual temperature of 8.7°C. The soil has developed on schist, sandstone, and quartzite and is classified as a Cambisol (IUSS, 2006). Loess was mixed by solifluxion and cryoturbation with primary rock weathering products (Platerboze, 1970).

Major soil characteristics are presented in Table III-1. Soil texture was sandy under the mor and silty under the moder. The Mn content was lower in soil under the mor than under the moder. The content of weatherable minerals, as estimated by their total reserve in bases (Herbillon, 1986), was lower in soil under the mor (20-59 $cmol_c.kg^{-1}$) than under the moder (95-172 $cmol_c.kg^{-1}$).

2. Sampling and analysis of forest floors

On each site, we sampled forest floors in December 2005 after leaf fall (Photoboard II). Forest floors were divided into horizons and subhorizons (Oln, Olv, Of1, Of2, Of3, and Oh) according to the definitions of Brêthes *et al*. (1995): Oln (plant remains), Olv (poorly fragmented), Of (fragmented) and Oh (dark humified organic matter). We collected each horizon or subhorizon separately from Oln to Oh on quantitatively 1 m^2 surface representing forest floor morphology on the site.

Three horizons (Olv, Of2, and Oh) from each forest floor, representing three stages of litterfall decomposition (Wardle, 1993), were analysed. Total elemental concentrations (Ca, Mg, K, Na, Mn, Al, Fe and P) were carried out by calcination (950°C) and lithium metaborate/lithium tetraborate fusion (Chao and Sanzolone, 1992) and measured by inductively coupled plasma atomic emission spectrometry (ICP-AES) (Jarell Ash). The C and N concentrations were measured by gas chromatography after dry combustion (1800°C) with a NC soil analyser (FLASH EA 1112 Series, Interscience, Thermo). We measured the concentration of lignin and cellulose by acid-detergent extraction (Van Soest, 1963).

3. Incubation, chemical analyses of water extracts, and C-mineralization

Forest floor samples were neither dried nor rewetted before the incubation. An equivalent of 10 g dry mass of Olv and Of2 horizons and an equivalent of 30 g dry mass of Oh horizon were incubated in separate PVC vessels (diameter = 160 mm, height = 200 mm). In each vessel, we applied a treatment with Mn or without Mn (control). For the Mn treatment, we added 250 mg Mn . $kg_{\text{dry matter}}^{-1}$, which is a realistic Mn^{2+} concentration in acid soil (Titeux, 2005).

Mn^{2+} was applied by aspersion of a $MnSO_4$ solution (1.5 mmol.l^{-1}) with a 3:1 ratio between solution volume and dry mass sample equivalent, involving no field capacity excess. For the control treatment, the same volume of demineralised water was added to the vessels. We put two plastic cups into each vessel, one with 1 mol.l^{-1} NaOH for CO_2 measurement following Eq. III-1 and one with water to balance NaOH-related dehydration:

$$NaOH + CO_2 \leftrightarrow Na_2CO_3 + H_2O \qquad \textbf{Eq. III-1}$$

The vessels were then hermetically sealed. Initial CO_2 content was measured in a closed vessel without a sample. All vessels were incubated in triplicate for 28 days at 20°C. Each week, vessels were opened to measure the CO_2 production by back titration of NaOH with HCl (1 mol.l^{-1}) and replace the cup of NaOH (1 mol.l^{-1}) solution. At the end of the incubation period, a 2 g (equivalent dry mass) subsample was extracted with 100 ml demineralised water from each vessel. Water extracts were filtered through a 0.45 µm pore size Whatman filter and analysed. We determined dissolved organic carbon (DOC) concentration using a carbon analyser (Dohrman DC-180) and Mn^{2+} concentration by ICP-AES.

In this study, CO_2 releases were cumulated over the incubation period. DOC and CO_2 releases were defined by the ratio between the amount of DOC and CO_2 (milligrams of C) released during the incubation period and the initial amount of C (grams) in the corresponding subsample.

III. Results and Discussion

1. Forest floor characteristics

Thickness (millimetres) and C-concentration (grams per square meter) of the forest floor horizons sampled are presented in Table III-2. Mor forest floor was thicker than moder forest floor (105 and 47 mm, respectively). In the mor forest floor, the Of horizon was thick (40 mm) and the morphology evolved from a squamous structure in its upper part to a fibrous structure in its lower part. According to field observations, Of and Oh horizons from the moder forest floor exhibited more signs of biological activities than Of and Oh from the mor forest floor, e.g. faecal pellets and the presence of minerals in the Oh horizon likely because of bioturbation with the underlying Ah horizon (Kruyts *et al.*, 2004). High Na, K, Fe and Al concentrations in the moder Oh horizon supports this hypothesis (Table III-3).

Table III-2: Thickness and C-stock of forest floor horizons.

	Horizon						Forest floor
	Oln	Olv	Of1	Of2	Of3	Oh	
a. Mor forest floor							
Thickness (mm)	20	10	10	20	10	35	105
C-stock ($g.m^{-2}$)	306	294	441	360	630	2282	4313
b. Moder forest floor							
Thickness (mm)	20	5	5	10	5	2	47
C-stock ($g.m^{-2}$)	197	155	225	254	328	135	1294

Mor forest floor horizons exhibited higher Ca concentration and lower Mn concentration than moder forest floor horizons (Table III-3). Cationic concentrations within tree leaves are influenced by geological, edaphical and climatical conditions as well as by physiological status (Albert and Pescoller-Tiefenthaler, 1992).
Here, the larger Mn concentration in the moder forest floor horizons (Table 3) could be partly explained by the larger Mn concentration in the moder site soil (Table 1). This was not the case for the Ca concentrations. The C concentration in the Oh horizon was much lower in the moder forest floor (25 %) than in the mor forest floor (43 %). The values of C/N and C/P ratio were higher in mor forest floor horizons (Table III-3). The moder Olv horizon exhibited a significantly higher lignin concentration (44.1 % ± 0.5) than mor Olv horizon (37.7 % ± 2.4; $P = 0.0048$, n = 3). Lignin concentration decreased by 9.7 % from the Olv to Oh horizon in the mor forest floor, while it decreased by 21.9 % in the moder forest floor (Figure III-1).

As lignin is usually considered to be a litter quality index (Melillo *et al.*, 1982), we can infer that the lower the lignin concentration, the greater the litter "biodegradability". In spite of the greater lignin concentration in litterfall, the moder forest floor exhibited by definition more effective decomposition dynamics: lower C/N and C/P, lower C accumulation, and stronger biological activity. This contradiction might be related to the high N and/or Mn concentrations in litterfall, which may have improved the decomposition dynamics of this moder forest floor (Berg, 2000).

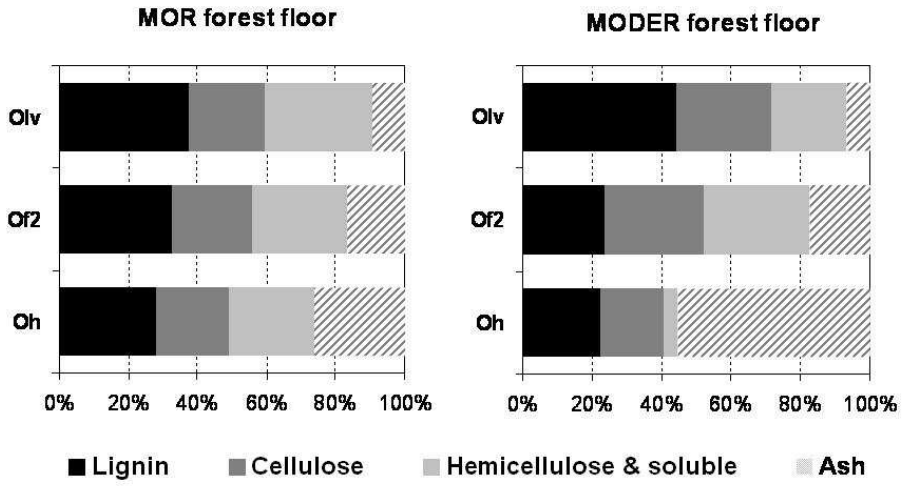


Figure III-1: Proportional biochemical composition of selected forest floor horizons (percentage of dry mass) obtained by acid detergent extraction (Van Soest, 1963).

Table III-3: Composition of selected forest floor horizons.

Horizon	C	N	P	C/N	C/P	Lignin	Cellulose	Ca	Mg	K	Na	Mn	Al	Fe
		%				(%), mean ± SD					$g.kg^{-1}$			
a. Mor forest floor														
Olv	45	1.9	0.16	23	281	37.7 ± *2.4* *b*	21.8 ± *2.6* *b*	8.1	0.9	2.0	0.4	0.8	1.0	0.9
Of2	43	2.1	0.16	21	269	32.7 ± *3.8* *c*	23.3 ± *0.6* *b*	4.4	0.5	1.4	0.5	0.4	2.4	3.1
Oh	43	2.3	0.15	19	287	28.0 ± *3.4* *cd*	20.9 ± *1.0* *bc*	2.6	0.7	2.4	0.7	0.1	5.7	6.0
b. Moder forest floor														
Olv	46	2.3	0.22	20	209	44.1 ± *0.4* *a*	27.3 ± *0.7* *a*	4.2	0.9	1.7	0.1	1.9	0.4	0.4
Of2	40	2.2	0.20	18	200	23.5 ± *0.2* *de*	28.7 ± *1.5* *a*	2.4	0.6	2.0	0.3	1.0	4.1	2.9
Oh	25	1.5	0.22	16	114	22.2 ± *1.1* *e*	18.2 ± *1.8* *c*	1.4	1.2	6.3	1.1	0.4	20.1	13.2
						P <0.0001	P =0.0006							

Note: Percentages are percentages of dry mass. For lignin and cellulose concentrations, means followed by different letters are significantly different from one another at the $P < 0.05$ level according to Fisher's least significant difference test (n = 3).
The detection thresholds of ICP-AES for cation concentrations are below 10 %.

2. Effects of Mn addition on C release

DOC release from moder horizons is greater than DOC release from mor horizons (Table III-4). As DOC is mostly released by lignin degradation (Sjöberg *et al.*, 2003), we assume that ligninolysis is more developed in the moder than in the mor forest floor. We observed no significant difference in DOC release in water extracts from control and Mn-treated horizons, except for the Oh horizon of the mor forest floor where DOC release from control treatment (0.35 mg C . g C^{-1} ± 0.04) showed a higher value than Mn treatment (0.25 mg C . g C^{-1} ± 0.01) (P = 0.0161) (Table III-4). The Mn^{2+} addition effect on DOC production could, in this case, be balanced by a parallel effect on DOC mineralization and/or on DOC solubilisation (Kalbitz *et al.*, 2006).

Carbon mineralization was slightly but significantly greater in the Mn treatment than in the control treatment of the Olv horizon of both forest floors (+ 10 %: mor, P = 0.0080; moder, P = 0.0111). In the Of2 horizon from the mor, the Mn-treated horizon released significantly more CO_2 than the control horizon (+ 25 %, P = 0.0007). In the Of2 horizon from the moder and Oh horizon from both forest floors, no effect of Mn addition was observed (Table III-4).

Since CO_2 release is used as an index of biological activity (Christ and David, 1996), we can assume that the Mn addition promoted biological activity, but only in some horizons (Figure III-2). This shy response of CO_2 release to Mn^{2+} addition could be explained by different factors: (i) physico-chemical conditions, (ii) forest floor type, and (iii) organic matter decomposition stage. Indeed, the addition of Mn as mobile Mn^{2+} did not guarantee its availability for the catalytic cycle of the manganese peroxidase once it met environmental conditions. Manganese evolves under several forms in soil according to pH and oxidoreduction conditions (Graham *et al.*, 1988). The Mn^{2+} concentration in water extracts from Mn-treated material was higher than the Mn^{2+} quantity in water extracts from the control material (Table III-5).

This increase in Mn^{2+} concentration was considerably greater in water extracts from mor horizons (+200 to 800 %) than those from moder horizons (+30 to 70 %). We also observed lower pH in water extracts from mor than in water extracts from moder, in the corresponding horizon. These results agree with the pE-pH diagram of Mn (Lindsay, 1979) where the preferential form of Mn at low pH is Mn^{2+}. Water extracts from the Olv horizons exhibited a higher pH (> 6) than water extracts from the Of2 and Oh horizons. According to the pE-pH diagram of Mn (Lindsay, 1979), the form of Mn at this pH could be Mn^{2+} but also manganese oxides (e.g. in the form of MnO_2 and Mn_2O_3), which are very stable. Thus, Mn immobilisation by oxidation could have limited the effects of the Mn^{2+} addition in the Olv horizons.

In agreement with Davey *et al.* (2007), who concluded that lignin degradation could take place early in the sequence of litterfall decomposition, we observed in our experiment that the Mn addition was already able to trigger ligninolysis in the Olv horizons. However, the strongest impact of the Mn addition was observed in the Mor Of horizon (increase in CO_2 release by 25 %), suggesting that physico-chemical conditions were more favourable to Mn^{2+} availability and/or that ligninolysis was more developed in this horizon. Actually, in the first stages of decomposition (Olv), microorganisms preferably consume easily available C (labile C) (Aber *et al*, 1990). Then, when the labile C source is depleted, lignin degradation is required to provide additional C source and allows microorganisms to access cellulose and hemicellulose embedded in the lignin matrix (Hammel, 1997). Considering that the Oh horizon consisted mainly of substances with more oxidized lignin whose path of decay differs from that of intact lignin, we observed no influence of the Mn addition in the Oh horizon

Table III-4: Carbon release by forest floor horizons.

	CO_2 (mg C. g C^{-1}), mean ± SD			**DOC** (mg C. g C^{-1}), mean ± SD		
	Olv	Of2	Oh	Olv	Of2	Oh
a. Mor forest floor						
Mn	6.33 ± 0.17 a	1.19 ± 0.03 a	0.60 ± 0.01a	1.13 ± 0.35 a	0.40 ± 0.04 a	0.25 ± 0.01 b
Control	5.70 ± 0.14 b	0.94 ± 0.04 b	0.60 ± 0.04 a	0.78 ± 0.20 a	0.38 ± 0.01 a	0.35 ± 0.04 a
P	*0.0080*	*0.0007*	*0.8818*	*0.2062*	*0.3819*	*0.0161*
b. Moder forest floor						
Mn	11.95 ± 0.28 a	5.86 ± 0.17 a	1.10 ± 0.01 a	5.22 ± 1.54 a	2.55 ± 0.50 a	0.20 ± 0.01 a
Control	10.77 ± 0.36 b	5.60 ± 0.22 a	1.01 ± 0.06 a	5.05 ± 1.05 a	2.02 ± 0.50 a	0.28 ± 0.05 a
P	*0.0111*	*0.1854*	*0.0803*	*0.3815*	*0.2633*	*0.1224*

Note: Mn and control represent the treatments applied on forest floor horizons: $MnSO_4$ solution and demineralised water, respectively. For each forest floor horizon, means followed by different letters are significantly different at the $P < 0.05$ level according to a Student *t* test (n = 3).

Table III-5: pH and Mn^{2+} concentration in forest foor horizon extracts.

	pH, mean ± SD			**Mn^{2+}** (mmol. kg $_{dry\ mass}^{-1}$), mean ± SD		
	Olv	Of2	Oh	Olv	Of2	Oh
a. Mor forest floor						
Mn	5.4 ± 0.5 a	4.7 ± 0.2 a	3.9 ± 0.0 a	0.09 ± 0.03 a	0.04 ± 0.01 a	0.03 ± 0.00 a
Control	6.3 ± 0.4 a	4.8 ± 0.1 a	4.2 ± 0.2 a	0.01 ± 0.01 b	0.01 ± 0.00 b	0.01 ± 0.00 b
P	*0.0995*	*0.5815*	*0.0861*	*0.0495*	*0.0177*	*<0.0001*
b. Moder forest floor						
Mn	6.1 ± 0.1 a	4.7 ± 0.0 a	4.5 ± 0.0 a	0.05 ± 0.03 a	0.24 ± 0.01 a	0.04 ± 0.00 a
Control	6.3 ± 0.3 a	4.7 ± 0.1 a	4.5 ± 0.0 a	0.03 ± 0.01 a	0.14 ± 0.03 b	0.03 ± 0.01 a
P	*0.1995*	*0.3081*	*0.5894*	*0.2670*	*0.0064*	*0.4906*

Note: Mn and control represent the treatments applied on forest floor horizons: $MnSO_4$ solution and demineralised water, respectively. For each forest floor horizon, means followed by different letters are significantly different at the $P < 0.05$ level according to a Student *t* test (n = 3).

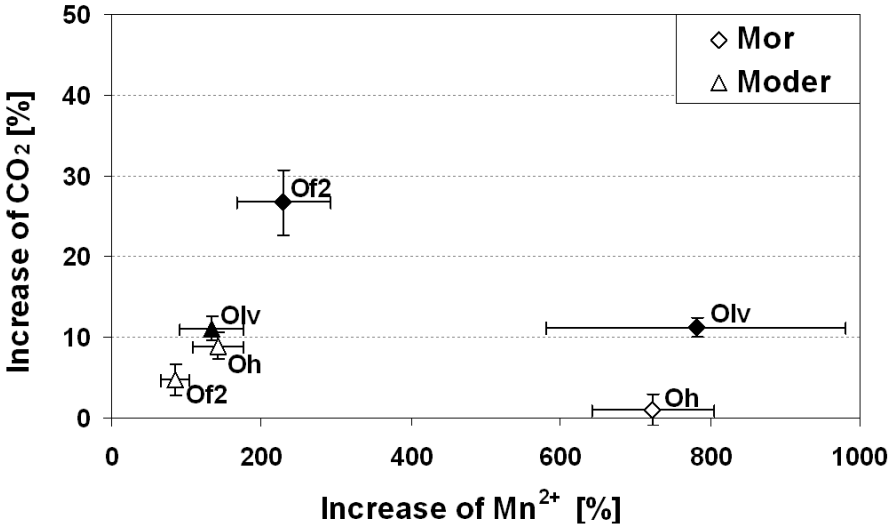


Figure III-2: Increase in CO_2 between treatments (Mn treatment compared with control treatment) *versus* increase in Mn^{2+} in water extracts between treatments. The points are presented with their standard deviations. The points where the increase in CO_2 release is significant are solid.

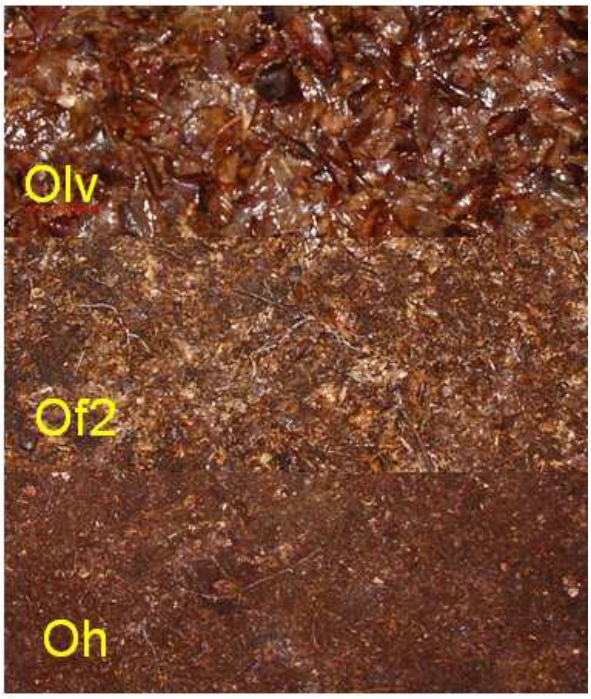
3. Conclusion

The impact of Mn^{2+} addition depends on the forest floor type but also on the organic matter decomposition stage (forest floor horizon). In Of horizons, there was an increase in CO_2 release only in the mor forest floor (Table III-4). The influence of Mn^{2+} additions was proportionally stronger in the mor forest floor horizons than in the moder forest floor horizons. We assume that it could be linked to lower initial Mn concentrations of the mor forest floor horizons and/or lower pH in the mor forest floor extracts (Table III-5). We suggest that Mn concentration could be limiting in the mor forest floor and not in the moder forest floor. We conclude that the effect of Mn^{2+} addition on forest floor decomposition is limited because of interaction with others factors and/or because the Mn concentration already suffices. This experiment highlights the contribution of Mn to the stimulation of forest floor decomposition and supports that Mn concentration could be also considered as an indicator of substrate quality due to its influences on C release.

Sampling site of Transinne



Forest floor horizons sampling in December 2005. The moder studied in the Part III was sampled in Transinne in the Belgian Ardennes (50°11'31"N, 4°59'03"E).



Selected horizons from moder forest floor

Part VI
Chapter 1

Influence of manganese on beech leaf litter decomposition during *in situ* incubation

Abstract

Litter decomposition is of crucial importance for sustainable production in forest ecosystems. Pedoclimatic conditions and litter quality are the main factors controlling litter decomposition; in particular, manganese concentration could accelerate litter decomposition. Correlations between manganese concentration and litter decay rate were previously reported and were explained by the role of Mn^{2+} in lignin degradation as a cofactor of the enzyme manganese peroxidase. However, the role of manganese has been little experimentally tested yet.

This study aims to assess the decomposition of beech (*Fagus sylvatica* L.) leaf litters experimentally enriched in manganese (0.9 to 17.0 $mg.g^{-1}$) using a litterbags experimental design (500 days of *in situ* incubation). Litter decomposition was estimated by mass loss measure and by analysis of remaining materials and litter leachates. Our results showed a positive influence of manganese on carbon decay and a modification of dissolved organic carbon (DOC) quality (increase in hydrophobic fraction) and of inorganic nitrogen forms (NH_4^+ or NO_3^-). We explained these results by an enhanced ligninolysis leading to an increase in dissolved polyphenolic compounds.

I. Introduction

Forest floor decomposition is a complex and fundamental process in the nutrient circulation in forest ecosystems and strongly impacts the global carbon and nutrient cycling. Nowadays, this topic is crucial in the context of carbon sequestration and greenhouse effect mitigation (Prescott, 2010).
The interest of scientists over litter decomposition has grown steadily since the 1980s. On one hand, it focus on the use of different litter quality indexes as litter decay predictors (e.g. C/N ratio, Anderson, 1973; essential nutrients such as N and P, Melin, 1930; Millar *et al.*, 1948; lignin concentration, Fogel and Cromack, 1977; lignin/N ratio, Melillo *et al.*, 1982). On the other hand, researchers propose models for describing the litter decomposition dynamics (Berg and Staaf, 1980; Coûteaux *et al.*, 1998; Louisier and Parkinson, 1976) and the calculating of a limit value by using asymptotic functions, which can provide an estimation of the carbon sequestration (Berg and Ekbohm, 1991; Berg *et al.*, 1996; Davey *et al.*, 2007).

Berg *et al.* (1996) were the first researchers to show a positive correlation between the initial manganese (Mn) concentration in litter and the levels of limit value. This link could be explained by the positive influence of Mn on lignin degradation: manganese peroxidase (MnP), an enzyme of the lignin degrading system secreted by the white-rot fungi, oxidizes Mn^{2+} to highly reactive Mn^{3+} ions, which penetrate the lignin structure and oxidize phenolic units (Hammel, 1997; Hofrichter, 2002; Perez and Jeffries, 1992). Perez and Jeffries (1992) also suggest that Mn promotes lignin degradation by stabilizing the lignin degrading enzymes and by enhancing their production.

More recently, several studies highlighted a positive correlation between the rate of forest floor decomposition (litter annual mass loss) and the Mn concentration in litterfall for Norway spruce (*Picea abies* (L.) Karst.), Lodgepole pine (*Pinus contorta* var. *contorta)* and Scots pine (*Pinus silvestris* L.) in late stage of decomposition (Berg *et al.*, 2007), for common oak (*Quercus robur* L.) (Davey *et al.*, 2007) and for a mix of

leave and needle litters in early stage of decomposition (Heim and Frey, 2004). Berg *et al.* (2010) even concluded that Mn concentration in pine needle litter may be used to predict the accumulation of humus and carbon sequestration and prospectively, that the role of Mn in litter decomposition among ecosystems and litters of different genera would deserve further attention.

The influence of Mn addition on forest floor decomposition has not been experimentally measured so far, except in our exploratory study (Trum *et al.*, 2011a – Part III) where we added Mn^{2+} on three horizons (Olv, Of2 and Oh) of two distinct forest floors. We observed an increase in CO_2 release depending on the forest floor type and on the organic matter decomposition stage. The effects of Mn^{2+} addition were probably limited by its availability mainly ruled by pH and oxidoreduction conditions.

In this paper, we studied the decomposition of beech (*Fagus sylvatica* L.) leaf litters experimentally enriched in Mn during an *in situ* incubation of 500 days using a litterbag experimental design. For a given site, climate and litter quality, we hypothesized that differences of Mn concentration in leaf litters would influence their decomposition dynamics. On this purpose, litter decomposition during *in situ* incubation was assessed by measuring the mass loss, the evolution of chemical and biochemical composition of decomposing litters and the release of dissolved organic carbon (DOC) and inorganic nitrogen (NO_3^- and NH_4^+).

II. Materials and methods

1. Pre-treatment of beech leaves

- Site of sampling

In July 2008, branches of mature beech (diameter of 1-2 cm) and 2-year-old beech seedlings were collected in a beech stand (*Fagus sylvatica* L.) located in the Bois de Lauzelle (50°40'52"N, 4°36'15"E) where the climate is humid temperate, with 835 mm mean annual rainfall and 9.4°C mean annual temperature. The beech stand of about 100 years is established on a dystric cambisol (IUSS, 2006) derived from quaternary loess. This soil is strongly acid and pH (H_2O) value is below 4.5 in all horizons. Major soil characteristics are given in greater details in Table IV-1. The beech stand forest floor is defined as a *moder* (Brêthes *et al.*, 1995).

We chose the beech (*Fagus sylvatica* L.) to carry out this experiment because it is a predominant tree species in temperate forests and it has one of the poorer litter qualities among the common European deciduous tree species (Figure IV-1) (Hättenschwiler, 2005).

Table IV-1: Major soil properties of the dystric cambisol (Bois de Lauzelle).

Horizon	Depth cm	pH	C_{tot}	N_{tot}	P_{tot}	C/N	TRB[a]	Mn
			% of DM [b]				$cmol_c\ kg^{-1}$	$g\ kg^{-1}$
Oh	1.5 of thickness	3.8	23.6	1.1	0.11	21	71	0.95
Ah	0 - 15	4.0	5.0	0.4	0.02	12.5	104	0.45
Bw	15 - 40	4.5	1.2	0.2	0.07	6	107	0.37

(a) The total reserve in bases (TRB) is the sum of total Ca, Mg, K and Na concentrations expressed in $cmol_c\ kg^{-1}$ and is an estimation of the content of weatherable minerals (Herbillon, 1986).
(b) DM is the abbreviation of dry matter.

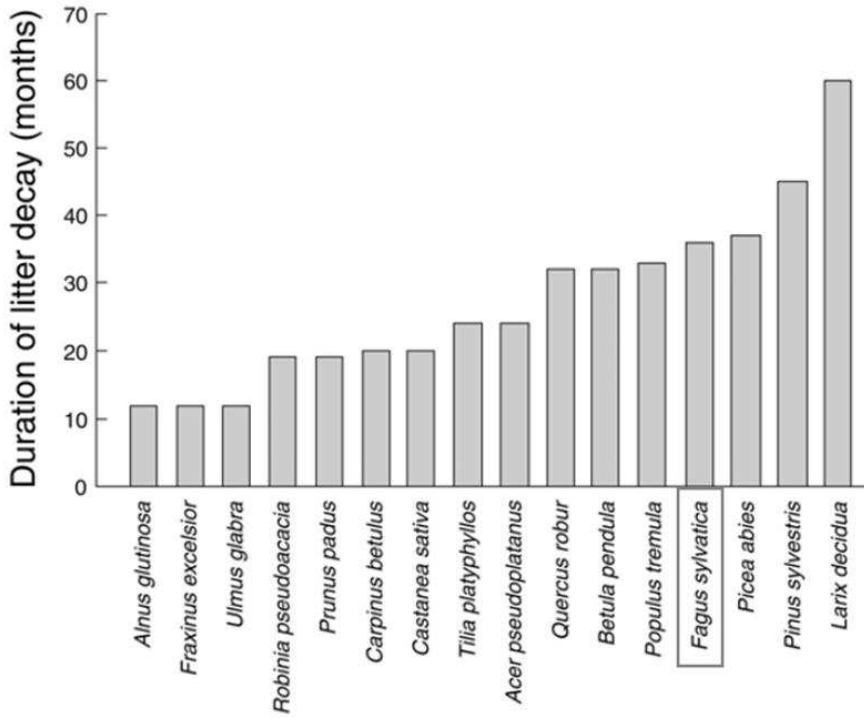


Figure IV-1: Average duration of decomposition of leaf litter from different European tree species exposed in the field under identical environmental conditions (from Hättenschwiler, 2005).

- Mn enrichment

In the laboratory, Mn concentration in beech leaves was experimentally adjusted by two distinct procedures: (i) dipping of mature beech branches in Mn solution and (ii) growing of beech seedlings in hydropony.

(i) Beech branches were in Mn solution (0, 0.5, 1 and 2.5 mM $MnSO_4$) for 10 days. Mn was transported through capillary action and accumulated in leaves (Loneragan, 1988).

(ii) Beech seedlings were grown in hydropony during 80 days under different Mn levels (0, 0.25, 0.5 and 1 mM $MnSO_4$) in a growth chamber with 448 µE m^{-2} s^{-1} photon density flux for 13h per days, 80% relative humidity and 19/13°C of day/night temperatures. The composition of the nutrient solution was: 1 mM $Ca(NO_3)_2$, 0.5 mM KCl, 0.5 mM K_2SO_4, 0.025 mM $MgCl_2$, 0.025 mM $MgSO_4$, 0.05 mM NaH_2PO_4, 80 µM H_3BO_3, 80 µM FeEDTA, 0.8 µM $ZnSO_4$, 0.8 µM $CuSO_4$ and 0.8 µM $(NH_4)_6Mo_7O_{24}$ (Ingestad, 1971). The nutrient solution was renewed every 3 weeks.

The obtained Mn concentrations in leaves [$mg.g^{-1}$] were the following:

	B-Mn0*	B-Mn1	B-Mn2	B-Mn3
Branch leaves	2.1	2.4	3.2	6.6
	S-Mn0	S-Mn1	S-Mn2	S-Mn3
Seedling leaves	0.9	5.7	12.8	17.0

* Abbreviated name for treatments

2. Litterbag experiment

Beech leaves were gently removed by hand from branches and seedlings. Green litter samples were obtained by enclosing leaf samples in litterbags made of nylon net with an upper mesh size of 2.4 mm (allowing access of micro- and meso-fauna) and a lower mesh size of 0.5 mm (preventing the loss of leaf fragments) (Berg and Laskowski, 2006).

Developed in the 1960s, the litterbag method remains the most commonly used technique for examining litter decomposition by measuring mass loss and estimating decomposition rates (Prescott, 2010). We have to keep in mind that this method presents several drawbacks (underestimation of decomposition and over-simplification of decomposition process) but stays the most convenient for comparative study (between Mn treatments in this study) (Wider and Lang, 1982).

There were two types of litterbags: (i) about 2.5 g (equivalent dry weight) of branch litter were enclosed in 18*18 cm^2 litterbag, which represent a quarter of annual litterfall on this surface; (ii) about 0.5 g (equivalent dry weight) of seedling litter was enclosed in 10*10 cm^2 litterbag. The size of litterbags was large enough to prevent "pillowlike" effect. In December 2008, litterbags were inserted below the Ol layer of a mor forest floor in undisturbed areas (Forêt de Soignes). We chose this stand because the conditions were not optimal for litter decomposition given that a mor forest floor developed (Brêthes *et al*., 1995). Table IV-2 presents the main characteristics of this stand. Litterbags were placed in a randomized design in at minimum two locations in the stand to avoid pseudoreplications (Webster, 2007). For each treatment, there were 12 samples of branch litter in each of three locations and 6 samples of seedling litter in each of two locations.

Samples were taken after 120 (t_1), 300 (t_2) and 500 (t_3) days of exposure in the field. At each sampling event and for each treatment, 12 branch litter litterbags and 4 seedling litter litterbags were collected and placed in individual plastic bags to minimise litter loss and avoid dehydration.

In the laboratory, plant remains such as mosses and roots were removed of each litterbag and remaining materials was dried to a constant mass at 60°C. Organic matter was estimated by difference between dry mass and ash content measured at 450°C for correcting mineral contaminations (Idol *et al*., 2002). Mass loss was measured as the difference in weight of residual organic matter at each sampling time relative to the corresponding initial mass.

$$MassLoss = 100 - \frac{(DM_{t_i} - Ash_{t_i})}{(DM_{t_0} - Ash_{t_0})} * 100 \ [\%] \qquad \textbf{Eq. IV-1}$$

Table IV-2: Selected characteristics of the mor forest floor where the litterbags were inserted (Forêt de Soignes, Bruxelles, Belgium) (data from Titeux (2005) and Trum *et al.* (2011a)).

Localisation	50°48'04" N, 4°28'04" E
Climate	Humid temperate 835 mm mean annual rainfall 9.4°C mean annual temperature
Soil	Podzol (IUSS, 2006) derived from tertiary sandy sediments. The content of weatherable minerals, as estimated by their total reserve in bases (TRB) (Herbillon, 1986) ranged from 20 to 40 $cmol_c\ kg^{-1}$.
Forest floor	Classified as a mor according to Brêthes *et al.*, 1995. Thickness of 105 mm (Oln, 20 mm; Olv, 10 mm; Of1, 10 mm; Of2, 20 mm; Of3, 10 mm; Oh, 35 mm). C-concentration of 4313 C-g m^{-2}.

3. Chemical analysis of litters and leachates

The chemical composition of litters was analysed before incubation (t_0) and after each sampling event (t_i). Carbon and N concentrations were measured by gas chromatography after dry combustion (1800°C) with a NC soil analyser (analyser FLASH EA 1112 Series, Interscience, Thermo). Total elemental concentrations (Ca, Mg, K, Na, Mn, Al, Fe and P) were carried out by calcination (450°C) and digestion with HNO_3, HF and $HClO_4$ and measured by inductively coupled plasma atomic emission spectrometry (ICP-AES, Jarell Ash). Ash concentrations were determined by combustion at 450°C for 24h. We measured the concentration of lignin and cellulose in leaf litters by acid-detergent extraction (Van Soest, 1963).

For each collected litterbag of branch litter only, soluble compounds were extracted from a 0.5 g (equivalent dry weight) subsample with demineralised water (0.5 g : 50 ml) using a batch method with 1 h agitation. After filtration of leachates through 0.45-µm pore size Whatman filters, we determined dissolved organic carbon (DOC)

concentration (C-mg.C-g^{-1}) using a Carbon Analyser (Dohrman DC-180), absorbance (at 280 nm) using UV/VIS spectrometer UNICAM8625, Mn^{2+} concentration (µg.g^{-1}) by ICP-AES, inorganic nitrogen (NH_4^+ and NO_3^-) concentration ($_N\mu mol._{DM}g^{-1}$) using HPLC (Dionex LC20 with IONPAC AS 11 columns). The specific molar absorptivity was defined by the ratio between the absorbance (at 280 nm) and the DOC concentration (C-g.C-g^{-1}) (Dilling and Kaiser, 2002; Simonsson *et al.*, 2005) and was expressed in cm^{-1}.

4. Statistical analysis

o Exponential model of decay

Litter decomposition rates were calculated by using the most commonly used function in litter decomposition studies, the single exponential decay function (Olson, 1963; Wider and Lang, 1982).

$$y_t = y_0 * e^{-kt} \qquad \textbf{Eq. IV-2}$$

Where y_t is the mass remaining at time t, y_0 is the initial mass, t is the time (number of days spent *in situ*) and *k* is the decomposition rate.
This model (Eq. IV-2) was linearised by a logarithmic transformation of the variables. Also, the intercept value was fixed given that the initial value y_0 was experimentally known.

$$\ln\left(\frac{y_t}{y_{0\,fixed}}\right) = -k * t \qquad \textbf{Eq. IV-3}$$

Equation IV-3 was fitted for litter mass and carbon amount of each treatment using JMP (SAS Institute, 2009) and PROC REG in the SAS software (SAS Institute, 1999). To compare the slope (*k*) between two models of different treatments (B or S-Mn0, Mn1, Mn2 and Mn3), we used a Student test.

The Student test statistic is:

$$T = \frac{|k_1 - k_2|}{\delta^2 \sqrt{\frac{1}{\delta_1^2} + \frac{1}{\delta_2^2}}} \sim St_{(n_1 + n_2 - 2)} \text{ where } \delta^2 = \frac{(n_1 - 1)\delta_1^2 + (n_2 - 1)\delta_2^2}{(n_1 - 1) + (n_2 - 1)}$$

The litter decay rate (k) estimation obtained from the single exponential model is based on the assumptions that all the material will be decomposed at the same decomposition rate. This model gives generally a good fit for the early stage of decomposition (Kurz-Besson *et al.*, 2005).

- Effects of manganese treatment and incubation period

Given that the factor "location" had not a significant effect, significance level of Mn treatments (B or S-Mn0, Mn1, Mn2 and Mn3) and litter type (branch or seedling litter) effects were assessed by a nested ANOVA2 (model: type, treatment(type)). When only one type of litter was considered, significance level of Mn treatments and incubation period (t_1, t_2 and t_3) effects were assessed by ANOVA2 (model: treatment, period, treatment*period). Furthermore, the effect of Mn treatment was compared for each period separately with the Fisher's least significant difference test (LSD). All these statistical analysis were performed with PROC GLM in the SAS software (SAS Insittute, 1999).

III. Results

1. Composition of beech leaves

Either for branch leaves or seedling leaves, there was no variation in chemical composition with the Mn enrichment. The characteristics of the undecomposed branch and seedling leaves (at t_0) are presented in Table IV-1. Both leaves had similar carbon, lignin and cellulose concentrations. Branch leaves exhibited larger N concentration and C/P ratio, lower P concentration and C/N ratio, larger values of Al, Fe and Na concentrations and lower values of Ca and Mg concentrations than seedling leaves.

Table IV-1: Chemistry of undecomposed leaves of *Fagus sylvatica* L.

		Leaf type		
		Branch leaves	Seedling leaves	*P*
C	%*	45.5 *±0.7*	44.7 *±0.5*	0.1101
N	%	2.2 *±0.2*	1.9 *±0.1*	0.0243 *
P	%	0.025 *±0.003*	0.161 *±0.019*	< 0.0001 ***
C/N		21.1 *±1.5*	23.7 *±0.8*	0.0194 *
C/P		1854 *±218*	281 *±4*	< 0.0001 ***
Lignin	%	45.5 *±9.0*	35.0 *±4.2*	0.0792
Cellulose	%	36.7 *±13.0*	28.8 *±1.9*	0.7194
Soluble+Hemicel.	%	13.6 *±6.8*	31.5 *±11.0*	0.0217 *
Ash	%	4.2 *±2.3*	4.7 *±1.0*	0.7010
Ca	mmol.kg^{-1}	178.9 *±16.1*	356.4 *±91.7*	0.0089 ***
Mg		58.4 *±3.0*	83.8 *±10.1*	0.0029 ***
Na		52.5 *±8.6*	3.1 *±0.7*	< 0.0001 ***
K		215.8 *±29.6*	215.2 *±64.0*	0.9859
Al		3.5 *±0.6*	1.7 *±0.4*	0.0020 ***
Fe		4.3 *±0.6*	3.0 *±0.7*	0.0393 *

Note: For each characteristic, means of tree and seedling leaves are significantly different at the $P < 0.05$ (*) or $P < 0.01$ (***) level according to a Student *t* test (n=4).
(*) C, N, P, lignin, cellulose, soluble + hemicellulose and ash concentrations are expressed as percentage of dry weight.

The Mn concentrations reached in seedling leaves (0.9 – 17.0 $mg.g^{-1}$) were larger than those reached in branch leaves (2.1 – 6.6 $mg.g^{-1}$). The *transfer coefficient* between Mn concentration in dipping solution and Mn concentration reached in leaves was higher for seedling leaves than for branch leaves (16.3 *vs* 1.9) (Figure IV-2).

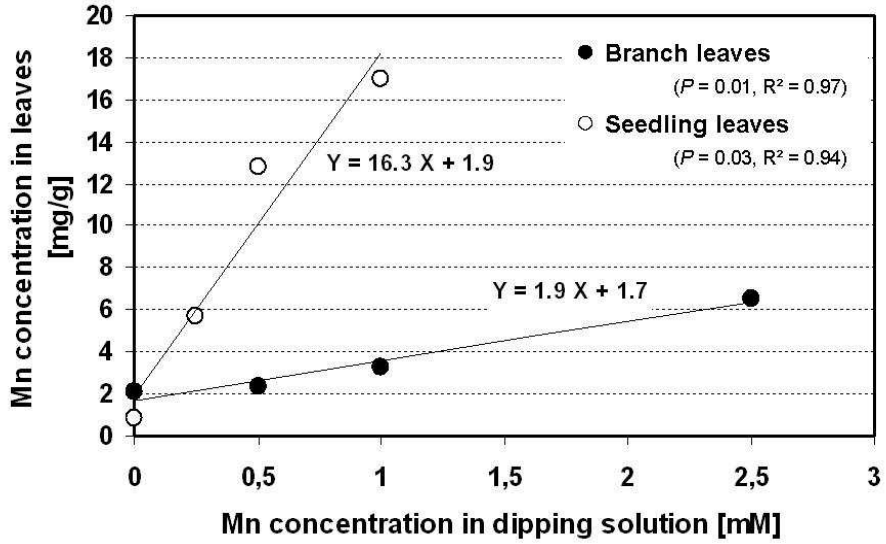


Figure IV-2: Manganese concentration in beech litters subjected to different treatments ($mg.g^{-1}$).

2. Assessment of decomposition rate

For the two first periods (t_1 and t_2), no significant differences of mass loss were observed between the treatments (t_1, $P_{treatment(type)}$ = 0.1977; t_2, $P_{treatment(type)}$ = 0.2679). For the last period (t_3), the larger mass loss were obtained for the B-Mn1, S-Mn2 and S-Mn3 treatments ($P_{treatment(type)}$ = 0.0419) (Table IV-2). For both leaf litters, significant decreases in remaining mass were observed between t_1 and t_3, except for the S-Mn0 and S-Mn1 seedling litters.

Table IV-2: Remaining mass (%) obtained for each treatment at the last period (t_3) (mean ± *SD*).

(P = 0.0419)	B-Mn0	B-Mn1	B-Mn2	B-Mn3
Branch litter	68 ±13 *abc*	57 ±11 *c*	71 ±9 *ab*	71 ±9 *ab*
	S-Mn0	S-Mn1	S-Mn2	S-Mn3
Seedling litter	80 ±13 *a*	69 ±20 *ab*	56 ±21 *bc*	56 ±16 *bc*

Note: Means followed by different letters are significantly different from one another at the $P < 0.05$ level according to Fisher's least significant difference test.

For the two first periods, significant differences of remaining C between litter types were observed (leaves quantity initially enclosed in litterbags was reported to 1g): remaining C of branch litters < remaining C of seedling litters (P_{type} =0.0274) for t_1 period; remaining C of branch litters > remaining C of seedling litters (P_{type} < 0.0001) for t_2 period.
For the two first periods, no significant differences of remaining C between the treatments were observed: remaining C ranged 0.39 to 0.58 g for t_1 period ($P_{treatment(type)}$ = 0.3752), from 0.18 to 0.33 g for t_2 period ($P_{treatment(type)}$ = 0.1085). For the last period (t_3), S-Mn0 and B-Mn2 litters exhibited the largest remaining C while B-Mn1, S-Mn2 and S-Mn3 exhibited the lowest ($P_{treatment(type)}$ = 0.0305).
For each treatment of both leaf litters, we observed significant remaining C differences between the t_1 and t_3 period except for the S-Mn0 seedling litters.

Given that it exists a strong correlation between the remaining mass (%) and the remaining C (g) (r = 0.91 $P < 0.0001$) (Figure IV-3), the carbon decrease over time could be modelled by the single exponential model of decay (Table IV-3).

Whatever the litter types, this model suited correctly to our data as (i) the estimated decay rate (k) were significant ($P < 0.0020$) and (ii) the determination coefficient (R^2) were high (from 0.69 to 0.87).

Whatever the litter type, there were significant differences of decay rate (k) between the treatments (Table IV-3). According to the Akaike information criteria (AIC), a linear relation was sufficient to adequately model the link between decay rates and Mn concentrations in litter (Figure IV-4).

Litter half-lives ($t_{1/2}$), the time necessary to reach 50% of remaining C if the decay rate is considered constant with time, ranged from 555 (B-Mn1) to around 750 days for branch litters and from 388 (S-Mn2) to 998 (S-Mn0) for seedling litters (Table IV-3).

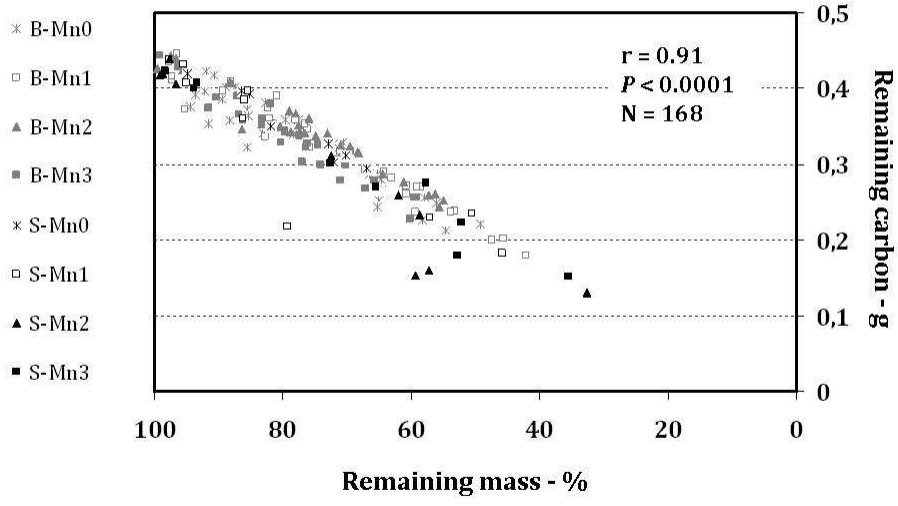


Figure IV-3: Pearson's correlation coefficient between remaining mass (%) and remaining C (g). The initial litter quantities enclosed in litterbags was reported to 1 g for each litter type.

Table IV-3: Estimation of decay rate for remaining C according to the exponential model $\ln(\frac{C_{ti}}{C_{t0}}) = -k * t$ where C_{t0} is fixed.

	Carbon decay constant *k* [d^{-1}]					R^2	N	$t_{1/2}$	
	Estimate (*P*)			95% confidence intervals				days	years
Branch litter									
B-Mn0	0.0011	b	*(<0.0001)*	0.0001	0.0012	0.87	36	653	1.8
B-Mn1	0.0012	b	*(<0.0001)*	0.0010	0.0015	0.80	31	555	1.5
B-Mn2	0.0009	cd	*(<0.0001)*	0.0007	0.0010	0.81	32	780	2.1
B-Mn3	0.0010	bc	*(<0.0001)*	0.0008	0.0011	0.84	30	711	1.9
Seedling litter									
S-Mn0	0.0007	d	*(0.0018)*	0.0003	0.0010	0.72	9	998	2.7
S-Mn1	0.0011	abcd	*(0.0015)*	0.0006	0.0017	0.69	10	614	1.7
S-Mn2	0.0018	a	*(0.0006)*	0.0010	0.0022	0.75	10	388	1.1
S-Mn3	0.0015	a	*(0.0003)*	0.0009	0.0020	0.83	9	461	1.3

Note: Litter half-lives ($t_{1/2}$) is the time necessary to reach 50% of carbon loss ($t_{1/2} = 0.691/k$).

Whatever the litter type, carbon decay constant (*k*) with different associated letters are significantly different from one another at the $P < 0.05$ level according to the slope comparison test of Student.

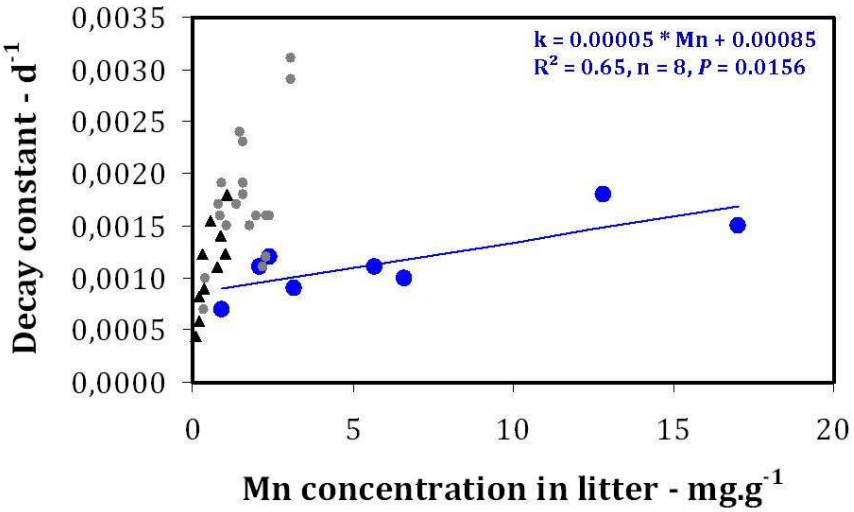


Figure IV-4: Computed decay rates (*k*) in function of the Mn concentration in litters - (●) our data, (●) data from Davey *et al*. (2007) and (▲) data from Heim and Frey (2004).

3. Litter quality changes

Branch litters lost about 50 percent of the initial lignin quantity (0.45 g at t_0, if litter quantities initially enclosed in litterbag was reported to 1 g) during the field incubation. At the end of the experiment, there were no significant differences of remaining lignin quantities (g) between treatments for branch litter (Table IV-4).
Whatever the treatment, lignin remaining quantities decreased significantly with time. The remaining lignin (g) was positively correlated with the remaining C (g) ($r = 0.83$, $P = 0.0008$).

Seedling litters lost a lower percentage of initial lignin quantity (0.35 g at t_0); the largest lost at the end of the incubation period was observed in S-Mn2 litter (28.5 %). Lignin remaining quantities were lower in S-Mn2 and S-Mn3 than in S-Mn0 and S-Mn1 seedling litters ($P < 0.0001$).

Table IV-4: Remaining lignin quantity (g) in branch litter obtained for each treatment and each period (mean ± *SD*). The initial litter quantities enclosed in litterbags was reported to 1 g.

Branch litter (n=4)

	B-Mn0	**B-Mn1**	**B-Mn2**	**B-Mn3**	$P_{treatment}$
t_1	0.44 ±*0.01* *ab*	0.44 ±*0.04* *b*	0.47 ±*0.00* *a*	0.41 ±*0.02* *b*	*0.1347*
	A	*A*	*A*	*A*	
t_2	0.37 ±*0.02* *ab*	0.34 ±*0.02* *b*	0.36 ±*0.03* *ab*	0.40 ±*0.04* *a*	*0.1357*
	B	*B*	*B*	*A*	
t_3	0.22 ±*0.03* *a*	0.24 ±*0.02* *a*	0.24 ±*0.06* *a*	0.21 ±*0.02* *a*	*0.7113*
	C	*C*	*C*	*B*	
P_{period}	*< 0.0001*	*< 0.0001*	*0.0007*	*< 0.0001*	

Note: For each sampling, means followed by different lowercase letters are significantly different from one another at the $P < 0.05$ level according to Fisher's least significant difference test. For each treatment, means followed by different uppercase letters are significantly different from one another at the $P < 0.05$ level according to Fisher's least significant difference test.

4. Leachates composition

The properties of branch litter leachates are showed in Table IV-5.

At t_1 and t_2, there were few significant differences in leachate properties between the Mn-treatments. At t_3, DOC concentrations were significantly largest in B-Mn2 and B-Mn3 branch litter leachates (P = 0.0274), the specific molar absorptivity in these leachates was the lowest for B-Mn0 branch litter and the largest for B-Mn3 branch litter.

At t_3, the NO_3^- concentrations were significantly lowest in B-Mn2 and B-Mn3 branch litter leachates (P = 0.0346) and the nitrification degree ($NO_3^-/(NH_4^+ + NO_3^-)$ ratio) was significantly largest in B-Mn0 branch litter leachates (P = 0.0104).

Table IV-5: Composition of leachates properties obtained for each treatment at the last period (t_3).

Treatment	DOC	Specific molar absorptivity	Mn^{2+}	NH_4^+	NO_3^-	Nitrification degree
	$Cmg.Cg^{-1}$	cm^{-1}	$\mu g.g^{-1}$	$N\mu mol.g^{-1}$	$N\mu mol.g^{-1}$	%
B-Mn0	10.0 ±*4.8* b	11.2 ±*2.8* b	2.3 ±*0.9* a	30.0 ±*13.0* a	5.6 ±*3.6* a	19 ±*10* a
B-Mn1	6.7 ±*2.1* b	20.6 ±*7.3* ab	1.7 ±*0.7* a	29.9 ±*4.7* a	3.2 ±*1.9* ab	9 ±*5* b
B-Mn2	17.7 ±*8.9* a	21.3 ±*12.6* ab	2.4 ±*1.4* a	28.4 ±*8.3* a	2.0 ±*0.7* b	7 ±*3* b
B-Mn3	11.5 ±*4.1* ab	31.6 ±*23.4* a	2.4 ±*1.1* a	29.8 ±*14.8* a	1.7 ±*0.4* b	6 ±*2* b
P	*0.0274*	*0.0694*	*0.5981*	*0.9928*	*0.0346*	*0.0104*

Means followed by different letters are significantly different fro one another at the $P < 0.05$ level according to Fisher's least significant difference test.

IV. Discussion

1. Mn concentration in leaves

With our experimental Mn enrichment of beech leaves, we obtained a plausible range of Mn concentrations (0.9 to 17.0 mg g^{-1}) in litter for natural ecosystems and comparable with those of the studies of Berg *et al.* (1996) (0.1 to 4.7 mg g^{-1}) and Davey *et al.* (2007) (0.4 to 3.1 mg g^{-1}). The linear relation between Mn concentration in leaf litter [mg g^{-1}] and Mn concentration in solution [mM] ($R^2 > 0.9$, $P < 0.05$) (Figure IV-2) allowed us to assert that the threshold for Mn toxicity was not exceeded in leaf litters.

In natural ecosystems, leaf age appeared to be the factor affecting the uptake of manganese (Kitao *et al.*, 2001). Manganese is readily transported from root to shoot through the transpiration stream, but not readily remobilized through phloem to other organs after reaching the leaves (Loneragan, 1988). Therefore, Mn concentration in leaves should reflect the cumulative amount of transpiration, leading to higher Mn concentration in seedling leaves as they stayed longer in contact with the solution enriched in Mn than the branch leaves stayed (80 *vs* 10 days).

2. Impact of Mn on litter decomposition

Whatever the Mn treatment, beech litters decomposed during the *in situ* incubation as the residual mass decreased significantly with the sampling time. At each period, either for branch litters or for seedling litters, results showed no mass loss differences between Mn treatments. However, the absence of significant differences in ANOVA analysis may be attributed to the large relative standard error (RSE, standard deviation divided by the mean and expressed as a percentage) of these data (1 to 37.5 %).

Instead of considering each sampling event separately, the comparison of decay rates parameters is simultaneously based on all sampling events. As a result, *degré de liberté* is higher, and differences between treatments are significant.

Manganese enrichment improved the litter decay rate (*k*) (Figure IV-4). In this figure, we compared our results with those observed by Davey *et al*. (2007) and Heim and Frey (2004) in which they also observed a significant positive correlation between Mn concentration in litter and decay rate. The range of Mn concentration in our leaf litters was much larger but the computed decay rates were lower. Other factors impacts litter decomposition; for example, Davey *et al.* (2007) studied litters of oak (*Quercus robur* L.) which usually develops a better decomposition dynamics than beech (*Fagus sylvatica* L.) (Figure IV-1).

This positive impact of Mn on litter decomposition could effectively be due to a positive impact of Mn on ligninolysis as we observed (i) for branch litters, a significant correlation between remaining carbon and remaining lignin and (ii) for seedling litters, a higher decrease in lignin concentration during the incubation of the Mn-richest litter (S-Mn3).

In the analysis of our data, we jointly considered the results of seedling and branch litters, but seedling litters likely presented a better *degradability* because of (i) a larger initial Ca concentration (356 *vs* 179 $mmol._{DM}kg^{-1}$) enhancing microbial and faunal processing (Hobbie *et al.*, 2006), (ii) smaller leaf size corresponding to more surface available for microbial attack (Cortez and Bouche, 1998) and (iii) a larger initial concentration of soluble and hemicellulose (31.5 % *vs* 13.6 %).

3. Impact of Mn on leachate properties

The composition of leachates extracted from branch litters proved to be sensitive to the Mn influence, overall after the last period of incubation (t_3).

The dissolved organic carbon (DOC) composition is complex and heterogeneous and contains as much as simple sugars as humic acids (Kalbitz *et al*., 2000). The main sources of DOC are C-labile (hydrophilic fraction) and product of the oxidative degradation of lignin (hydrophobic fraction) (Guggenberger, 1994). UV absorbance at 280 nm is a successful predictor of the aromaticity of DOC (Chin *et al*., 1994; Kalbitz *et al*., 2003). Thus, the specific molar absorptivity indicates the proportion of DOC hydrophobic components (Simonsson *et al.*, 2005). The larger DOC release by Mn rich branch litters at the last period (t_3) could be explained by enhanced ligninolysis. Indeed, the larger molar absorptivity of these leachates suggests that the additional DOC originates from lignin degradation.

At the last period (t_3), the release of nitrate and the nitrification degree were lower for the leachate of the Mn-richest litter (B-Mn3). It could be explained by an increase in nitrate consumption by microorganisms and/or by a depression of nitrification process due to an enhancement of ligninolyis and consequently due to an increase in dissolved polyphenolic compounds (Guggenberger, 1994). Indeed, the polyphenolic compounds (e.g. polyphenols, tannins) are involved in allelopathic interactions, which directly or indirectly reduce microbial activity, change microbial community and inhibit nitrification reaction (Kraus *et al.*, 2003; Schimel *et al*., 1996).

4. Conclusions

- We obtained beech litters of equal composition except Mn concentration by a simple and reliable method.
- Based on the composition of leachates extracted from branch litters, we suggest that Mn influences the quantity and the quality of DOC release, the release of inorganic nitrogen by an enhancement of ligninolysis.
- Field study results in some variability in the data because all the environmental parameters could not be controlled. To reduce this variability and precise the Mn influence, we propose a laboratory study focusing on analysis of leachate composition.

a. Sampling site of Lauzelle



Branches of mature beech tree and 2-year-old beech seedlings were collected in a beech stand located in the Bois de Lauzelle (50°40'52"N, 4°36'15"E).
The beech stand of about 100 years is established on a dystric cambisol (IUSS, 2006).



Beech stand
(*Fagus sylvatica* L.)



2-year-old beech seedling

b. Experimental Mn-enrichment

In the laboratory, Mn concentration in leaves was experimentally adjusted by two distinct procedures:

(i) Dipping of mature beech branches in Mn solution

(ii) Growing of 2-year-old beech seedling in hydropony



Mn solution
(0, 0.5, 1 and 2.5 mM $MnSO_4$)





The containers with seedlings were then placed in a growth chamber during 80 days under different Mn level.

c. Study site of Soignes

In December 2008, litterbags were inserted below the Ol layer of a mor forest floor in undisturbed areas.





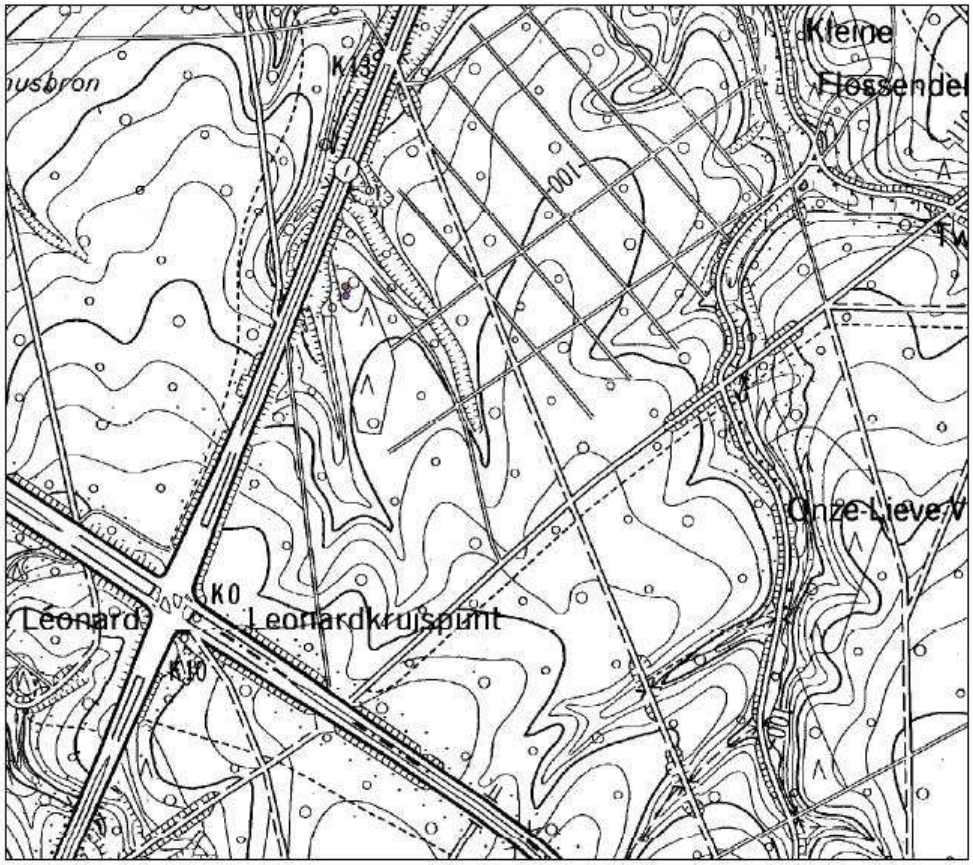

The sampling events took place after 120, 300 and 500 days of exposure in the field

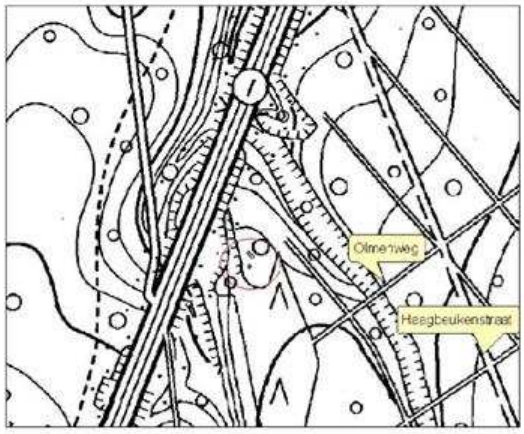
In the laboratory, plant remains were removed of each litterbags and remaining material was dried at 60°C






d. Litterbags setting in 3 zones of the study site of Soignes





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Part VI
Chapter 2

Influence of manganese on carbon released by decomposing beech leaf litters: a microcosm experiment

Abstract

Lignin concentration in organic residues is considered as a retarding factor of litter decomposition, a vital process in forest biogeochemical cycle. Manganese ion (Mn^{2+}) plays a key role in ligninolysis as it is essential for the catalytic cycle of manganese peroxidase, a central enzyme of the lignin degrading system. Correlations between Mn concentration and litter decay were previously reported. However, the role of Mn has not been experimentally tested yet.

Therefore, we experimentally adjusted Mn concentration in beech leaves with $MnSO_4$ solution. We investigated the impact of Mn on litter decomposition by incubating these beech leaves (Mn concentration from 0.9 to 17.0 $mg.g^{-1}$) for one year (early stage of decomposition) in laboratory under controlled conditions. We determined carbon release (CO_2 and DOC) and the change in litter composition during the incubation period. With increasing Mn concentration, litters exhibited an increase in total CO_2 release and a decrease in total DOC release whose origin preferentially was C-labile.

In the light of our results, we conclude that Mn concentration promotes complete mineralization of organic matter in the early stage of decomposition by improving biological activity. We did not conclude to an impact on ligninolysis probably because of the absence of specific ligninolytic communities in the laboratory microcosms.

I. Introduction

Decomposition of litters refers to the physical, chemical and biological processes involved in reducing litter to its elemental constituents. Litter decomposition has a major role in forest ecosystem functioning by influencing nutrient circulation. This complex ecological process is strongly controlled by two interacting factors, soil and climatic conditions and chemical composition of the litter, called *substrate quality* (Swift *et al*., 1979).

The control of decomposition rate by substrate quality had been broadly studied in the literature and focused on two main issues (Taylor *et al.*, 1989): (1) what properties of leaf chemistry or structure are responsible for differences in decomposition rate? (2) which of these properties can be the best summarized into a simple measure or index for predictive purpose?
Litter decomposition is controlled by a wide variety of litter chemical properties including the N concentration or the C/N ratio (Berg and Staaf, 1980), the P concentration or the C/P ratio, and the lignin concentration or the lignin/nutrient ratio (Melillo *et al.*, 1982). However, since lignin is held to be considered as a rate-retarding compound (Fogel and Cromack, 1977), lignin concentration remains a privileged substrate quality index and is considered to be the major factor in litter decay.

Lignin degradation is controlled by various factors such as N concentration (Keyser *et al.*, 1978), pH and oxygen concentration (Crawford, 1981). Moreover, Kerem and Hadar (1995) suggested that Mn had a positive influence on lignin degradation because of the functioning of the manganese peroxidase. This enzyme of the lignin degrading system, secreted by the white-rot fungi, oxidizes Mn^{2+} to highly reactive Mn^{3+} ions, which penetrate the lignin structure and oxidize phenolic units (Hammel, 1997; Hofrichter, 2002; Perez and Jeffries, 1992).

Recently, several studies (Berg *et al.*, 2007; Berg *et al*., 2010; Davey *et al*., 2007; Heim and Frey, 2004; Virzo De Santo *et al.*, 2009) highlighted positive correlations between the rate of litter decomposition and the Mn concentration in litterfall. These correlations depend on the litter type and on the decomposition stage. Among others, Davey *et al*. (2007) and Virzo De Santo *et al*. (2009) observed this type of correlation during the early stage of decomposition and concluded that the degradation of lignin could start immediately after litterfall.

These previously cited studies were only based on statistical analysis of existing decomposition data from natural ecosystems. In this paper, we generated purposely data by enriching beech leaves experimentally in manganese.

This study aims to consider the impact of Mn on litter decomposition during the early stage of decomposition. Beech (*Fagus sylvatica* L.) leaves of contrasting Mn concentrations were incubated in microcosms for about 350 days under laboratory controlled conditions. Litter decomposition was evaluating by CO_2 release (respirometry) and by dissolved organic carbon (DOC) obtained by leaching analysis.

II. Materials and methods

1. Pre-treatment of beech leaves

Cfr Part IV, Chapter 1, pages 85 – 87.
The obtained Mn concentration in beech leaves [$mg.g^{-1}$] were the following:

	B-Mn0*	B-Mn1	B-Mn2	B-Mn3
Branch leaves	2.1	2.4	3.2	6.6
	S-Mn0	S-Mn1	S-Mn2	S-Mn3
Seedling leaves	0.9	5.7	12.8	17.0

* Abbreviated name for treatments

2. Laboratory incubation in *microcosms*

Leaf samples of different Mn treatments were neither dried nor rewetted before the incubation.

i. For branch leaves and for each B-Mn treatment, an equivalent of 5 g dry mass of branch leaves were incubated in separate PVC vessels (diameter = 160 mm, height = 180 mm).
ii. For seedling leaves and for each S-Mn treatment, an equivalent of 1 g dry mass of seedling leaves was incubated in separate PVC vessels (diameter = 115 mm, height = 200 mm).

Leaves were incubated without additional microbial inoculums. We put two plastic cups into each vessel, one with 25 ml of NaOH (1M) for CO_2 measurement following Eq. IV-4 and one with water to balance NaOH-related dehydration.

$$NaOH + CO_2 \leftrightarrow Na_2CO_3 + H_2O \qquad \textbf{Eq. IV-4}$$

The vessels were then hermetically sealed. Initial CO_2 content was measured in a closed vessel without a litter sample. All vessels were incubated in triplicate at 20°C for 376 and 294 days, respectively for branch and seedling litters.

Several times during the incubation period (about every month), vessels were opened to measure CO_2 production by back titration of NaOH with HCl (1M) and replace the cup of NaOH (1M) solution. Moreover, about every fifty days, soluble compounds were extracted from a 0.25 g (equivalent dry weight) subsample of branch litter only with demineralised water (0.25: 50 ml) using a batch method with 1 h agitation. The distribution of respirometry measurements and water extractions during the incubation period is illustrated in Figure IV-5.

The term *microcosm* is generally used in ecology for any enclosure containing a small sample of "the real world" (Berg and Laskowski, 2006), in our case, a sample of leaf litter with microorganisms naturally inhabiting the leaf litter system. Thanks to this system, leaf litter decomposition took place under similar controlled conditions (i) to assert that differences among leaf litters investigated were mainly related to difference in their Mn concentration and (ii) to restrict variability in the data compared with that under field conditions.

3. Chemical analysis of litter

The chemical composition of leaf litters were analysed before (t_0) and after (t_f) the incubation period. Carbon and N concentrations were measured by gas chromatography after dry combustion (1800°C) with NC soil analyser (FLASH EA 1112 Series, Interscience, Thermo).
We measured the concentration of lignin, cellulose, soluble + hemicellulose, and ash in leaf litters by acid-detergent extraction (Van Soest, 1963).

For branch litters, total elemental concentration (Ca, Mg, K, Na, Mn, Fe and P) were performed by calcination (450°C) and digestion with HNO_3, HF and $HClO_4$ and measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Jarell Ash).

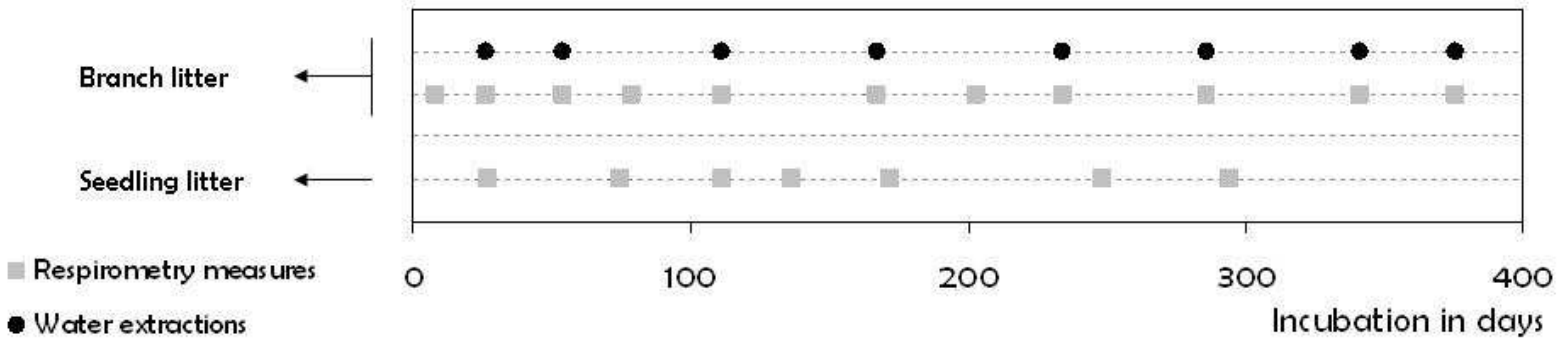


Figure IV-5: Calendar of respirometry measurements and water extractions on the incubation period

4. Analysis of branch litter leachates

After filtration of leachates through 0.45-µm pore size Whatman filters, we determined dissolved organic carbon (DOC) concentration (C-mg . Cg^{-1}) using a carbon analyser (Dohrman DC-180), absorbance (at 280 nm) using UV/VIS spectrometer UNICAM8625, Mn^{2+} concentration ($mg.g^{-1}$) by a flame atomic absorption spectrometer (Spectra AA-300 VARIAN) using a wavelength of 279.5 nm.

The specific molar absorptivity is defined by the ratio between the absorbance (at 280 nm) and the DOC concentration (g C.g C^{-1}) (Dilling and Kaiser, 2002; Simonsson *et al.*, 2005) and thus, was expressed in cm^{-1}.

In this study, releases by branch litters were cumulated over the incubation period. DOC and CO_2 releases were expressed by the ratio between the amount of DOC and CO_2 (mg) released during the incubation and the initial amount of C (g) in the corresponding sub-sample.

5. Statistical analysis

For each litter type, the differences between means were compared with one-way analysis of variance (ANOVA1) and Fisher's least significant difference, with a significant level of 0.05. Polynomial regressions were fitted to quantify the evolution of CO_2 release against the incubation time. All these statistical analysis were performed with PROC GLM and PROC REG in the SAS software (SAS Institute, 1999).

III. Results and discussion

1. CO_2 release

Cumulative CO_2 release (C-mg.C-g^{-1}) during the incubation period showed a different pattern according to the litter type (branch litter *vs* seedling litter):

i. For branch litters (Figure IV-6 a),

For each respirometry measure taken during the incubation period, cumulative CO_2 releases of B-Mn3 litters were significantly larger than release of B-Mn0 litters ($P < 0.0150$). For each B-Mn treatment, we modelled the cumulative CO_2 release evolution in function of the incubation time by a polynomial of degree 2 ($Y = a*X^2 + b*X + c$) (Figure IV-6 b). This model was the most suitable for our data among the linear and the exponential models according the Akaike information criterion (AIC), giving the relative goodness of fit of statistical models.

ii. For branch litters (Figure IV-6 c),

For each respirometry measure taken during the incubation period, cumulative CO_2 released by seedling litters of different S-Mn treatment followed the order S-Mn0 < S-Mn2, S-Mn3 < S-Mn1 ($P < 0.0200$). For each S-Mn treatment, cumulative CO_2 release reached a maximum plateau after 120 days. For comparison, we also modelled these data with a polynomial of degree 2 (Figure IV-6 d).

The two types of litters (branch and seedling litter) released more or less the same C quantity under CO_2 form (about 200 C-mg.Cg^{-1}) during the incubation period. However, this quantity was more rapidly released by seedling litters than by branch litters likely because of larger (soluble + hemicellulose) concentration constituting C-labile easily mineralizable.

Table IV-6 gives the coefficient of the polynomial of degree 2 models. Conceptually, the coefficient "a" of this model gives information about the time needed to reach the maximum of CO_2 release while the coefficient "b" gives information about the *slope* to reach this maximum. Branch litter models had a significant lower "a" coefficient than seedling litter models. Model for B-Mn0 treatment had the lowest "b" coefficient (Table IV-6).

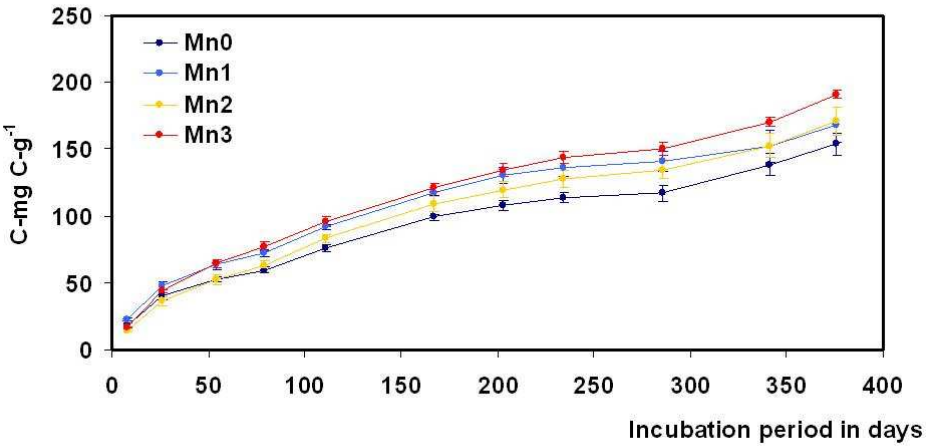
Since the measurement of respiration (CO_2 release) provides a useful index of biological activity (Christ and David, 1996), we can assume that biological activity was promoted in branch litters of higher Mn concentration (Figure IV-6 a). On the other side, we noticed that CO_2 released by seedling litters did not increase with Mn-concentration (Figure IV-6 c). Mn-richer seedling litters had a much higher Mn concentration (S-Mn2, 12.8; S-Mn3, 17.0 mg.g^{-1}) than in Mn-richer branch litters (B-Mn2, 3.2; B-Mn3, 6.6 mg.g^{-1}). These higher Mn concentrations may involve other mechanisms antagonizing CO_2 release.

Table IV-6: Significant polynomial regressions of cumulative CO_2 released by decomposing litters against incubation time.

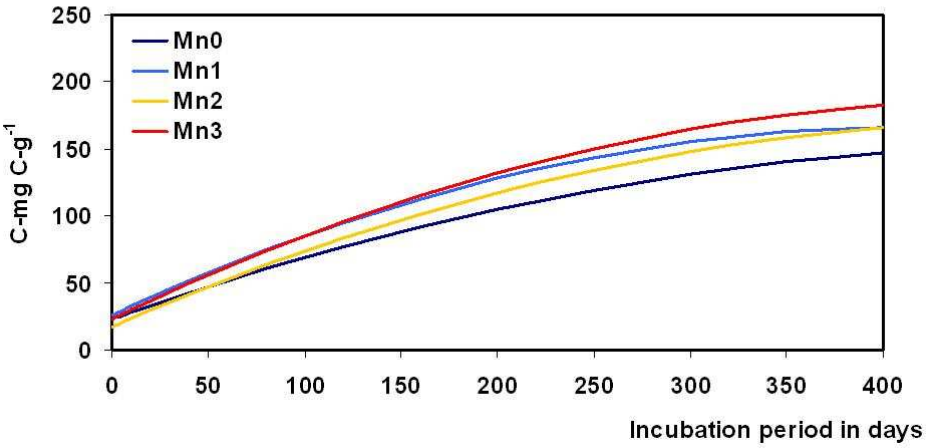
$Y = a*X^2 + b*X + c$ where Y is the cumulative CO_2 (mg C.g C^{-1}) released by litters and X is the incubation time (days). Models followed by different letters have coefficient significantly different at the $P < 0.05$ according to the Student *t* test (n=3).

Treatment	Polynomial model ($Y = a*X^2 + b*X + c$)			R^2	N
	- a	b	c		
B-Mn0	0.0005 (± 0.0001)	0.51 (± 0.04)	22.9 (± 3.0)	0.97	33
P	*<0.0001 c*	*<0.0001 b*	*<0.0001*		
B-Mn1	0.0008 (± 0.0001)	0.67 (± 0.04)	26.0 (± 3.3)	0.97	33
P	*<0.0001 b*	*<0.0001 a*	*<0.0001*		
B-Mn2	0.0006 (± 0.0001)	0.63 (± 0.04)	17.3 (± 3.2)	0.97	33
P	*<0.0001 bc*	*<0.0001 a*	*<0.0001*		
B-Mn3	0.0007 (± 0.0001)	0.69 (± 0.04)	23.1 (± 3.2)	0.98	33
P	*<0.0001 b*	*<0.0001 a*	*<0.0001*		
S-Mn0	0.0019 (± 0.0003)	0.83 (± 0.11)	72.4 (± 7.4)	0.85	21
P	*<0.0001 a*	*<0.0001 a*	*<0.0001*		
S-Mn1	0.0024 (± 0.0005)	1.06 (± 0.17)	112.4 (± 12.3)	0.78	21
P	*0.0002 a*	*<0.0001 a*	*<0.0001*		
S-Mn2	0.0019 (± 0.0005)	0.79 (± 0.19)	99.03 (± 13.0)	0.58	21
P	*0.0023 abc*	*0.0005 ab*	*<0.0001*		
S-Mn3	0.0020 (± 0.0004)	0.86 (± 0.14)	93.8 (± 10.0)	0.77	21
P	*0.0002 a*	*<0.0001 ab*	*<0.0001*		

(a) CO_2 release by branch litters - *observed data*



(b) CO_2 release by branch litters - *predicted data*



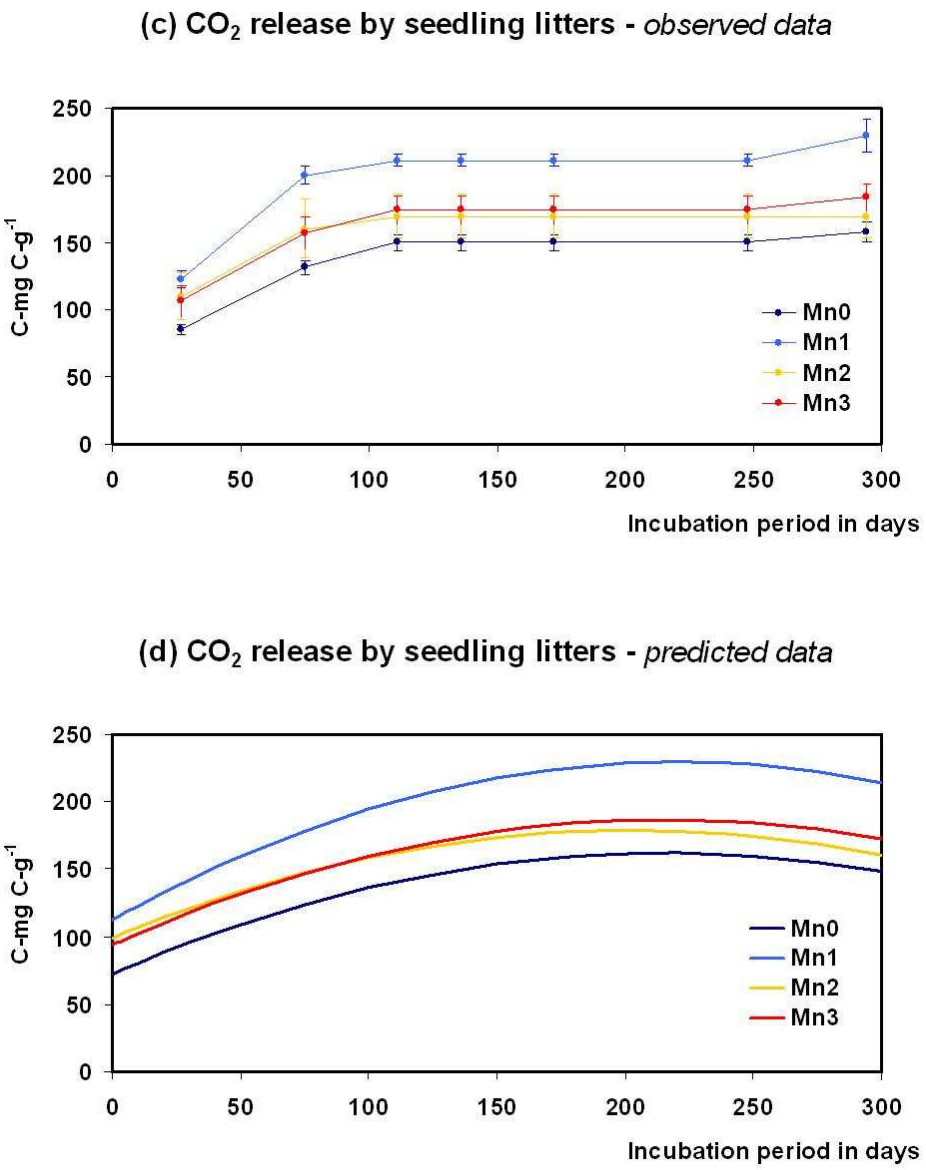


Figure IV-6: Cumulative CO_2 release (expressed in C-mg.C-g^{-1} during incubation in days). Error bars represent ± SD of each measure.

2. DOC release

Concerning leachate analysis (only for branch litters), cumulative DOC released by branch litter of different Mn-treatments followed the order B-Mn0 > B-Mn1 > B-Mn2 > B-Mn3 ($P < 0.0100$) (Figure IV-7).

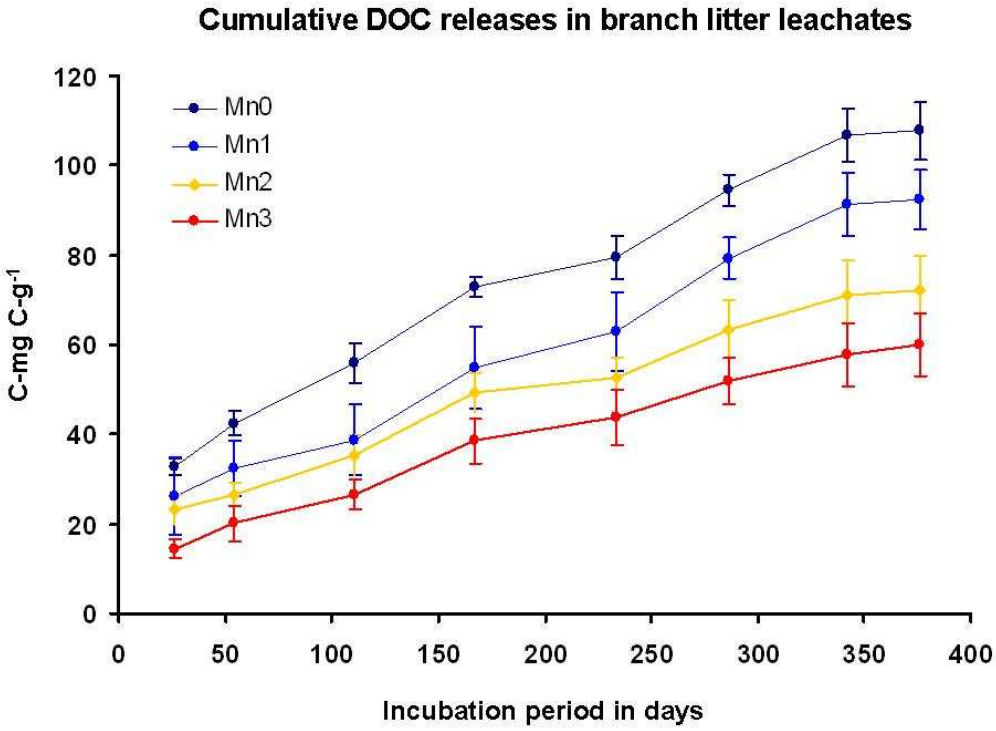


Figure IV-7: Cumulative DOC release (expressed in C-mg.C-g^{-1} during incubation in days). Error bars represent ± SD of each measure.

3. CO_2 and DOC releases

For each Mn treatment, the DOC/(CO_2+DOC) ratio (%) stayed mostly constant during the incubation and decreased with the Mn concentration level. The minimum and maximum values reached for this ratio during the incubation period are the following: Mn0, 41.1-45.2%, *P* = 0.0003; Mn1, 29.5-37.5%, *P* = 0.4689; Mn2, 29.1-38.9%, *P* = 0.0009; Mn3, 21.6-25.7%, *P*=0.7970.

The total releases of C-compounds computed over all the incubation period are presented in Table IV-7. B-Mn3 litter had the largest CO_2 release (*P* = 0.0092) and B-Mn0 litter had the largest DOC release (*P* = 0.0001). The DOC/(CO_2 + DOC) ratio of the global C-release over the incubation decreased with the Mn concentration level (*P* < 0.0001). The Mn-richer branch litters (B-Mn2 and B-Mn3) had a lower specific molar absorptivity than the Mn-poorer branch litters (B-Mn0) (*P* = 0.0557) (Table IV-7).

The DOC composition is complex and heterogeneous, the main source of DOC are labile C (hydrophilic products) and products of the oxidative degradation of lignin (hydrophilic products) (Guggenberger, 1994). UV absorbance at 280 nm is a successful predictor of the aromaticity of DOC (Chin *et al.*, 1994; Kalbitz *et al.*, 2003). Thus, the specific molar absorptivity indicates the proportion of DOC hydrophobic components (Dilling and Kaiser, 2005; Simonsson *et al.*, 2005). The DOC composition slightly varied with the Mn-treatment (*P* = 0.0557). Mn-richer litter (B-Mn2 and B-Mn3) leachates were poorer in aromatic components that B-Mn0 litter leachates (Table IV-7).

Table IV-7: Total releases of various compounds over the incubation period (from branch litters). For each compound, means followed by different letters are significantly different from one another at the $P < 0.05$ level according to Fisher's least significant difference test (n=3).

	CO2	**DOC**	**DOC + CO_2**	$\frac{DOC}{DOC + CO_2}$	**Specific molar absorptivity**	**Mn^{2+}**
	C-mg.C-g^{-1}			%	cm^{-1}	$mg.g^{-1}$
B-Mn0	158 ±*8*	108 ±*6*	262 ±*14*	41 ±*1*	36 ±*16*	0.13 ±*0.01*
	b	a	a	a	a	b
B-Mn1	168 ±*13*	92 ±*7*	261 ±*14*	35 ±*2*	21 ±*9*	0.24 ±*0.16*
	b	b	a	b	ab	b
B-Mn2	171 ±*10*	72 ±*8*	243 ±*17*	30 ±*2*	15 ±*5*	0.32 ±*0.26*
	b	b	a	c	b	ab
B-Mn3	191 ±*3*	60 ±*7*	251 ±*4*	24 ±*2*	11 ±*2*	0.59 ±*11*
	a	b	a	d	b	a
P	*0.0092*	*0.0001*	*0.3566*	*<0.0001*	*0.0557*	*0.0448*

4. Change in litter decomposition during the incubation period

The increase in C-mineralization of litters of richer Mn-concentration resulted in a larger decrease in C-concentration during the incubation period (Figure IV-8). Decomposition of litter materials is essentially accompanied by CO_2 production and therefore by a reduction in C resources (Albers *et al.*, 2004).

Whatever the litter type (except for B- and S-Mn0 treatment), N concentration in litters increased significantly (> +29%, $P < 0.0500$) during the incubation and we observed a significant decrease in C:N ratio (> -30%, $P < 0.0500$) (Figure IV-8). This increase in N concentration during the incubation period indicates that both litter types were still in the early stage of decomposition. Indeed, litter decomposition process is conceptually divided in two phases. In short, the first phase (early stage), soluble substances and labile compounds are rapidly decomposed and in the second phase (late stage), cellulose and lignin are slowly decomposed (Berg, 1986).

Regarding the N concentration in litter, this process results in a first step of net N-accumulation (immobilization phase) followed by a net release of N (mineralization phase) (Melillo *et al.*, 1989).
We observed no difference of lignin concentrations in litter during the incubation excepting for B-Mn3 branch litter and S-Mn0 seedling litter. Lignin concentration significantly decreased from t_0 to t_f for B-Mn3 branch litters (t_0, 45.5% ± 10.3% > t_f, 35.9% ± 2.1%, $P = 0.0241$) while it increased for S-Mn0 seedling litters (t_0, 35.0% ± 4.2% > t_f, 43.5% ± 4.1%, $P = 0.0241$).

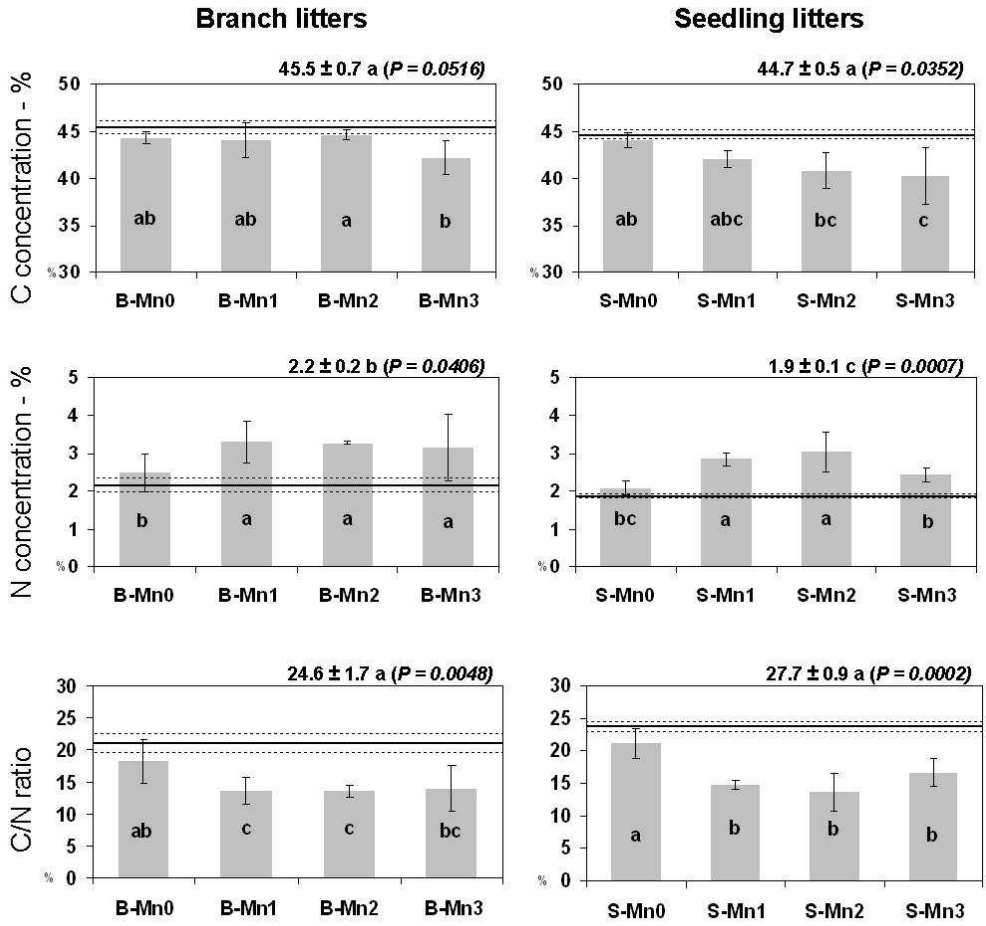


Figure IV-8: Carbon and N concentrations and C:N ratio of branch/seedling litters during the incubation period lasting 376 and 294 days, respectively for branch and seedling litters.

For each graphic, mean (n=3) followed by different letters are significantly different from one another at the $P < 0.05$ level according to Fisher's least significant difference test (the precise P value is indicated in the top right). For each graphic, the continuous horizontal line represents the initial corresponding measure, the dashed horizontal lines represent this measure ± SD. Error bars represent ± SD.

5. Conclusions

The results of seedling litters incubation did not allow us to draw clear conclusions about the impact of manganese on their decomposition.
However, the results of branch litters incubation suggest that Mn concentration affects C released by decomposing litters. Total carbon (CO_2 + DOC) released by Mn-treated branch litters stayed constant. The mineralized form (CO_2) was the privileged form of carbon release with increasing Mn concentration in branch litters. The decrease in DOC release with Mn-treatment could be due to an increase in biological (bacteria) activity, needing larger C sources.

These results, summarized in Figure IV-9, do not provide evidence that the Mn influence on the C-release pattern was due to an impact of Mn on ligninolysis (e.g. no clear decrease in lignin concentration, no increase in DOC concentration and no increase in specific molar absorptivity during the incubation period). Figure IV-9 showed that the effect seemed to be proportional to the Mn concentration.

Our study was an enclosed laboratory experiment, then, it was possible that ligninolytic fungi were not initially present in the microcosms. Nevertheless, we observed a change of C-release patterns (Figure IV-9) with increasing Mn concentration in litter. The Mn and/or the $SO_4^=$ enrichment could trigger bacterial activity as nutrients.

This experiment reminds that the Mn is foremost a minor element and an essential nutrient, and as such, it can promote microbial activity (expressed by C-mineralization). We did not observe an influence of Mn on lignin degradation, probably because of the absence of adequate ligninolytic fungi communities. This conclusion encourages the collaboration between soil scientists and microbiologists in litter decomposition studies.

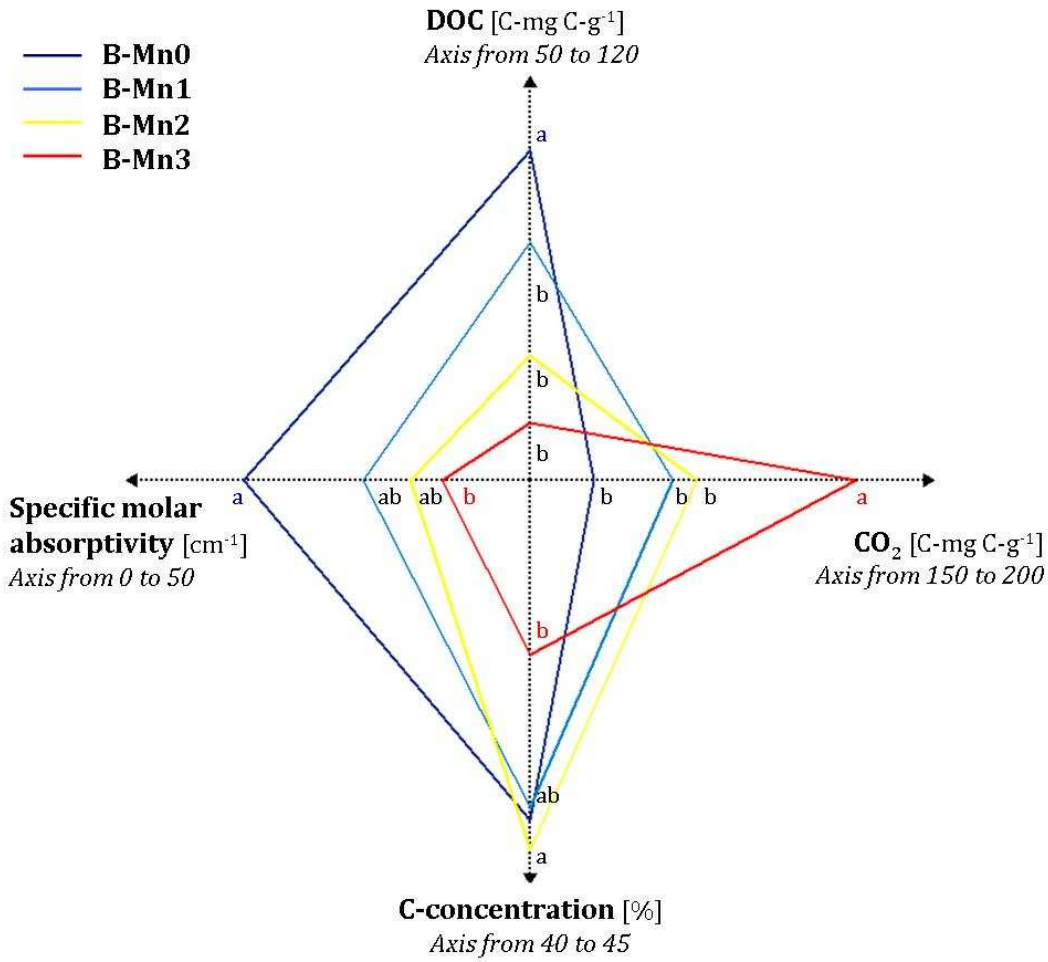


Figure IV-9: Summarized patterns of key characteristics of C-released from branch litters. For each properties, mean followed by different letters are significantly different from one another at the $P < 0.05$ level according the Fisher's least significant difference test.

Part VI
Chapter 3

Manganese effect on lignin degradation degree in beech leaf litters

Abstract

Lignin content exerts a major control on litter decomposition. Manganese may positively affect lignin degradation because of its role in the catalytic cycle of an enzyme of the lignin degrading system. This study aims to assess the effect of manganese concentration on lignin degradation degrees (computed with CuO oxidation products) of decomposing beech leaves experimentally enriched in manganese and incubated *in situ* for 500 days and *in vitro* for 300 days. At the end of incubation, the Mn rich litters exhibited a lower concentration in lignin units (VSC sum), a larger $(Ac/Al)_v$ and $(Ac/Al)_S$ ratios and surprisingly a lower S/V and C/V ratios. We conclude that the ligninolysis improvement by Mn is not observable in all the cases and is mediated by the biotic environment in which decomposition takes place.

I. Introduction

Litter quality controls organic matter decomposition in temperate forest ecosystems and thereby storage and cycling of nutrients and organic carbon. Its decomposition is mainly driven by lignin degradation (Fogel and Cromack, 1977). The recalcitrance of lignin is explained by its complex aromatic structure (i.e. heterogeneous phenylpropane units connected randomly by different types of bonds), which is directly related with its functions of protection and rigidity of plant tissues (Hammel, 1997). Therefore, only the white-rot fungi organisms are able to achieve the complete lignin degradation, referred to as enzymatic combustion (Kirk and Farell, 1987), by secreting unspecific and extracellular enzymes such as lignin peroxidase, manganese peroxidase (MnP) and laccase (Hammel, 1997; Hofrichter, 2002).

Recently, several studies (Berg *et al*., 2007; Berg *et al.*, 2010; Davey *et al*., 2007; Heim and Frey, 2004; Virzo De Santo *et al*., 2009) highlighted a positive correlation between the rate of litter decomposition and the Mn concentration of plant litter. Given that MnP is the key enzyme of the lignin degrading system (Hofrichter, 2002), this correlation could be explained by the positive influence of Mn on ligninolysis. Indeed, MnP oxidizes Mn^{2+} to Mn^{3+}, which penetrates the lignin structure and oxidizes phenolic units (Perez and Jeffries, 1992; Hammel, 1997; Hofrichter, 2002). Perez and Jeffries (1992) also suggested that Mn promoted lignin degradation by stabilizing the lignin-degrading enzymes and by enhancing their production. However, the influence of Mn on lignin degradation in leaf litters has not yet been evaluated.

Cupric oxide oxidation (CuO oxidation, Hedges and Ertel, 1982) is a technique widely used for lignin characterization and quantification in plants, sediments and soil organic matter (Kögel, 1986). This relatively mild oxidation yields a suite of single-ring phenol compounds which are indicative of lignin origin and degradation state (Thevenot *et al.*, 2010).

In this study, we examined the lignin parameters obtained by CuO method in decomposing beech (*Fagus sylvatica* L.) litters experimentally enriched in Mn. We hypothesized that these parameters indicate a more advanced state of lignin degradation in Mn-rich leaf litters compared to Mn depleted litter.

II. Materials and methods

1. Leaf litter samples

- Pre-treatment of beech leaves

Cfr Part IV, Chapter 1, pages 85-87
The obtained Mn concentration in beech leaves [$mg.g^{-1}$] were the following:

	B-Mn0*	B-Mn1	B-Mn2	B-Mn3
Branch leaf	2.1	2.4	3.2	6.6
	S-Mn0	S-Mn1	S-Mn2	S-Mn3
Seedling leaf	0.9	5.7	12.8	17.0

* Abbreviated name for treatments

- Incubations conducted on the beech leaves

Two types of incubation were conducted on these leaves:

1) Firstly, leaves were incubated *in situ* for 500 days in the field below the Ol layer of a mor forest floor using a litterbag experimental design.
   Cfr Part IV, Chapter 1, pages 87-89.

2) Secondly, leaves were incubated *in vitro* in separate PVC-vessels under laboratory controlled conditions (at 20°C, without any additional microbial inoculums).
   Cfr Part IV, Chapter 2, pages 111-112.

- Selection of decomposed leaf samples

The different samples used in this study are summarized in Table IV-8.

Table IV-8: Selected leaf litter samples for the experiment.

Sample	Leaf litter type	Incubation method	"Treatment"
1	Branch leaf litter (B)		(t_0) B-Undecomposed
2		*In vitro*	(t_f) B-Control
3			(t_f) B-Mn
4		*In situ*	(t_f) B-Control
5			(t_f) B-Mn
6	Seedling leaf litter (S)		(t_0) S-Undecomposed
7		*In vitro*	(t_f) S-Control
8			(t_f) S-Mn
9		*In situ*	(t_f) S-Control
10			(t_f) S-Mn

2. Leaf litter analysis

o Carbon and nitrogen concentrations

For each sample, C and N concentrations were measured by dry combustion (1800°C) with a NC soil analyser (FLASH EA 1112 Series, Interscience, Thermo).

o CuO oxidation products

Chemolytic analyses of lignin were conducted by the cupric oxide (CuO) oxidation method (Hedges and Ertel, 1982) as modified by Kögel and Bochter (1985). Samples were dried (40°C) and ground (< 0.75 mm). Briefly 50 mg dry mass of sample were oxidized with 250 mg of powdered CuO, 50 mg of $Fe(NH_4)_2(SO_4)_2.6H_2O$ and 10 ml of NaOH (2M). The oxidation was carried out in teflon vials under N_2 atmosphere for 2.5 h at 170°C. After cooling, the oxidation products were purified using a combination of washing, centrifugation and acidification (with HCl 6M). The separation and the quantification of the oxidation products were carried out by HPLC (waters 1525 binary HPLC pump, chromatographic conditions: isocratic eluant – 850/152/5 (% vol) H_2O/MeOH/H_3PO_4 25°C, 0.7 ml.min^{-1}).

The lignin-derived phenolic CuO oxidation products are presented in Figure IV-10. They were expressed as sum VSC, i.e. Vanillyl + Syringyl + Cinnamyl compounds (Kögel, 1986). The vanillyl compounds (V) are vanillic acid and vanillin, the syringyl compounds (S) are syringic acid and syringaldehyde and the cinnamyl compounds (C) are *p*-coumaric acid and ferulic acid. Their quantities (mg) were normalized to the carbon concentration (g C^{-1}) of each leaf litter. Indexes computed from these phenolic compounds provide excellent means of evaluating the quantity, the origin, the quality and the degradation state of lignin in environmental samples (Thevenot *et al*., 2010). In general, sum VSC, S/V and C/V ratio decrease upon lignin degradation whereas $(Ac/Al)_S$ and $(Ac/Al)_V$ increase.

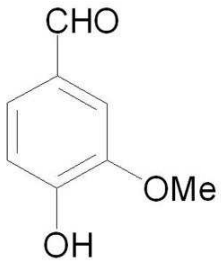
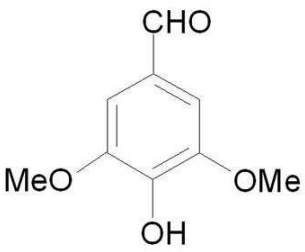
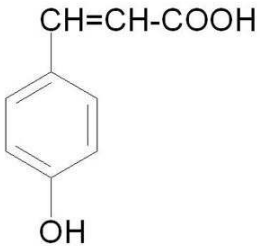
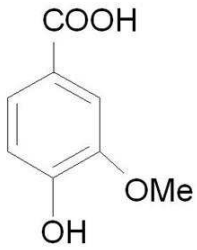
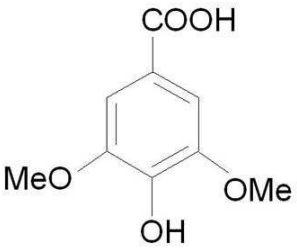
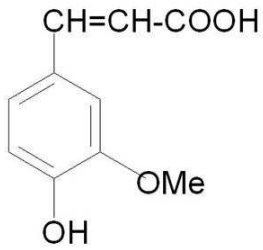
V compounds	S compounds	C compounds
		
(a) Vanillin	(c) Syringaldehyde	(e) *p*-coumaric acid
		
(b) Vanillic acid	(d) Syringic acid	(f) Ferulic acid

Figure IV-10: Determined products from alkaline CuO oxidation of lignin (from Kögel, 1986).

- Statistical analysis

For each combination of leaf litter (branch *vs* seedling) and incubation (*in situ vs in vitro*), the difference between means were compared with a Student *t* test. The two treatments considered were t_f *control* and t_f *Mn*. We chose a significant level of 0.10 to highlight the most different trends. Some correlation analysis were performed on the litter properties obtained at the end of the incubation. These statistical analyses were performed with PROC TTEST and PROC CORR in the SAS software (SAS Institute, 1999).

A principal component analysis (PCA) was carried out on our data (n=10) to investigate the relationships between the substrate quality indexes and the lignin parameters. Following the PCA, we performed as well a hierarchical cluster analysis using Euclidean distances and Ward's method to identify possible groups in our samples. These analyses were performed using the free-software R (version 2.12.1) with the plug-in factomineR.

III. Results and discussion

1. Effect of manganese concentration on lignin degradation

The sum of the oxidation products, defined as the sum of vanillyl (V), syringyl (S) and cinnamyl (C) units (Figure IV-10) and called VSC, provides an excellent estimator of intact lignin structural units concentration (Dignac *et al*., 2005).

The initial quantity of lignin (Van Soest lignin) was lower in seedling leaves than in branch leaves (35.5 ± 4.2 *vs* 45.5 ± 9.0, P = 0.0792). Moreover, the initial composition of seedling leaves (at t_0) exhibited a much larger VSC sum than branch leaves (P = 0.0068). Every of the three units constituting the VSC sum were significantly larger in seedling leaves than in branch leaves (V unit, P = 0.0203; S unit, P = 0.0015; C unit, P = 0.0660) (Table IV-9).

The decrease in VSC sum during the incubation period was significant only for *in situ* incubated Mn-treated seedling litters. These differences of VSC sum were explained by specific decrease of V, S and C units in branch and seedling litters. No significant correlation was observed between VSC sum and Van Soest lignin.

These shy results suggest that Mn concentration promoted ligninolysis only during the *in situ* incubation. The main difference between the two types of incubation concerns the temperature and moisture conditions and the variety of existing microbial communities. Indeed, the substrate quality affects the microbial community inducing the release of extracellular enzymes that degrade and modify the substrate. In turn, the substrate break products drive microbial succession (Fioretto *et al*., 2007). The microbial communities could evolve during the *in situ* incubation while they could not vary during the *in vitro* incubation as it was carried out in enclosed microcosms. Therefore, we hypothesise that the white-rot fungi involved in lignin degradation were absent of the incubation microcosms.

Table IV-9: Carbon, nitrogen and the oxidation products (VSC) concentrations in the samples.

	Incubation	Treatment	Carbon %	Nitrogen %	V mg.C-g^{-1}	S mg.C-g^{-1}	C mg.C-g^{-1}	VSC mg.C-g^{-1}
Branch litter		t_0 B-Undecomposed	45.5 ± 0.7	2.2 ± 0.2	17.7 ± 7.0	14.3 ± 8.2	7.7 ± 0.0	39.1 ± 15.2
	In vitro	t_f B-Control	44.3 ± 0.6 a	2.5 ± 0.5 a	13.5 ± 5.3 b	6.4 ± 4.8 a	3.0 ± 2.9 a	22.9 ± 12.2 a
		t_f B-Mn	42.2 ± 1.8 b	3.2 ± 0.9 a	21.9 ± 1.0 a	10.8 ± 1.1 a	5.5 ± 2.5 a	38.2 ± 4.4 a
		P	*0.0652* (n=3)	*0.1647* (n=3)	*0.0500* (n=3)	*0.1946* (n=3)	*0.3283* (n=3)	*0.1114* (n=3)
		t_0 B-Undecomposed	45.5 ± 0.7	2.2 ± 0.2	17.7 ± 7.0	14.3 ± 8.2	7.7 ± 0.0	39.1 ± 15.2
	In situ	t_f B-Control	41.6 ± 3.2 a	3.4 ± 0.6 a	26.3 ± 13.6 a	24.7 ± 1.7 a	4.5 ± 3.1 a	65.7 ± 4.4 a
		t_f B-Mn	42.9 ± 1.9 a	3.4 ± 0.4 a	20.9 ± 0.4 a	11.3 ± 3.9 b	5.9 ± 1.3 a	38.1 ± 5.0 b
		P	*0.1216* (n=12)	*0.3565* (n=12)	*0.5252* (n=3)	*0.0057* (n=3)	*0.4915* (n=3)	*0.0080* (n=3)
Seedling litter		t_0 B-Undecomposed	44.7 ± 0.5	1.9 ± 0.1	40.3 ± 16.7	42.1 ± 9.2	10.0 ± 2.5	92.4 ± 28.5
	In vitro	t_f B-Control	44.0 ± 0.8 a	2.1 ± 0.2 b	18.9 ± 12.8 a	14.4 ± 9.1 b	1.7 ± 1.2 b	35.1 ± 23.1 a
		t_f B-Mn	40.2 ± 3.0 b	2.4 ± 0.2 a	27.9 ± 0.3 a	30.2 ± 5.0 a	4.4 ± 0.1 a	62.5 ± 5.1 a
		P	*0.0978* (n=3)	*0.0741* (n=3)	*0.4271* (n=3)	*0.0891* (n=3)	*0.0891* (n=3)	*0.2429* (n=3)
		t_0 B-Undecomposed	44.7 ± 0.5	1.9 ± 0.1	40.3 ± 16.7	42.1 ± 9.2	10.0 ± 2.5	92.4 ± 28.5
	In situ	t_f B-Control	44.3 ± 1.7 a	2.9 ± 0.8 a	25.5 ± 5.2 a	22.8 ± 9.4 a	4.4 ± 0.2 a	52.7 ± 14.4 a
		t_f B-Mn	42.1 ± 0.9 b	2.2 ± 0.3 b	3.9 ± 1.4 b	9.6 ± 3.4 a	0.7 ± 0.3 b	14.3 ± 5.0 b
		P	*0.0279* (n=4)	*0.0587* (n=4)	*0.0300* (n=3)	*0.2033* (n=3)	*0.0046* (n=3)	*0.0706* (n=3)

Note: For each combination of litter and incubation types, means followed by different letters are significantly different from one another according to Student *t* test. Number of repetitions (n) varies depending on the substrate quality index and is indicated after the *P-value.*

2. Effect of manganese concentration on lignin characteristics during degradation

The ratios between S units and V units (S/V) and between C units and V units (C/V) are considered to be related to lignin degradation. During this process, S and C units are preferentially degraded compared to V units (Bahri *et al*., 2006). Therefore, the remaining part of the lignin molecules becomes enriched in V units and the S/V and C/V ratios decrease with increasing decomposition (Kögel, 1986).

According to the introductive hypothesis of this study (e.g. Mn concentration promotes ligninolysis), we expected that Mn-enriched litters (Mn) exhibit lower S/V and C/V ratios than the control. Our results were not in agreement with this hypothesis: we observed either no significant difference or higher ratios in Mn litters compared to control litters (Figure IV-11). They may be explained by the early phase of lignin degradation.

Bahri *et al*. (2008) modelled the degradation of maize milled enzymatic lignins (MMEL-lignins) in laboratory by a first order kinetic model with two compartments. They suggested that the initial phase of lignin degradation was rapid (mean residence time of 62 days) but only concern a tiny proportion of the initial MMEL-lignins (2.7%). The vast majority of the MMEL-lignins turn over was much slower with an estimated mean residence time of 25 years.

Several authors reported an increase in the ratio S/V during the initial phase of decomposition because of (i) a pronounced release of V compounds, probably from lignocellulose (Kögel, 1986) and of (ii) a preferential initial microbial attack of S compounds in angiosperm wood (Christman and Oglesby, 1971) and litter (Kögel, 1986).

The ratio between the acid and aldehyde forms for vanillyl and syringyl units (abbreviated $(Ac/Al)_V$ and $(Ac/Al)_S$, respectively) are also considered as indicators for lignin degradation.

These ratios provide a measure of the oxidation state of the lignin side chain (depolymerisation) (Hedges *et al.*, 1988). These ratios have been shown to increase upon lignin degradation in soils (Kögel-Knabner *et al.*, 1988).

For both incubations, the branch litters enriched in Mn exhibited an higher $(Ac/Al)_V$ ratio than the control ($P < 0.10$, Figure IV-11). For seedling litters, this was not the case. The $(Ac/Al)_S$ ratio showed similar results (Figure IV-11). Then, our data seem to suggest, that at least for branch litters, high Mn concentrations enhanced lignin degradation.

The values of $(Ac/Al)_V$ ratio were relatively large in comparison with those reported for arable and grassland soils (< 1) but were in the same range than those reported for forest soils (1.3 – 2.1, Kögel, 1986; Jolivet *et al.*, 2001). Guggenberger *et al.* (1994) suggested that such differences may be due to the fact that lignins in surface forest soils are more degraded than lignins in agricultural soils.

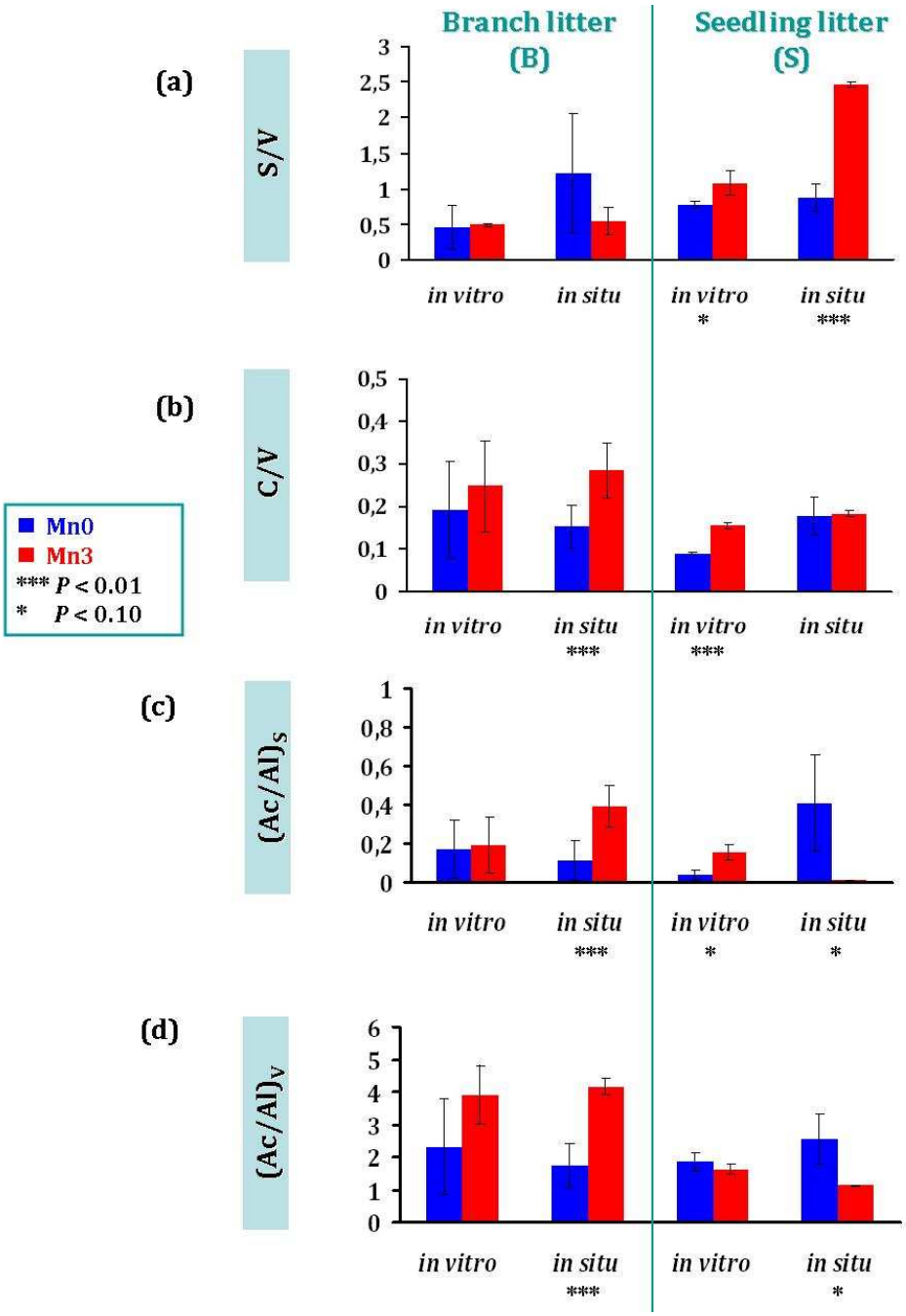


Figure IV-11: Lignin parameters computed from CuO oxidation products for each combination of litter and incubation types. For each combination of litter and incubation types, the *P-value* corresponds to a mean comparison according a Student *t* test. Error bars are ± SD.

3. Relationships between litter properties (PCA)

By studying our data with classical statistical analysis, it was difficult to draw clear conclusions. For this reason, we performed multivariate analysis to test if any trends emerge from our data.

Except the carbon and Mn concentration and the C/V ratio, the variables were relatively well represented by the two first dimensions of the PCA, given that the vectors representing them were close to the correlation circle (Figure IV-12a). These two dimensions explained 65% of the data.

The PCA explored the relations between these variables. Here, the PCA highlighted:

i. An axes representing the lignin degradation process with which the $(Ac/Al)_V$ and $(Ac/Al)_S$ ratios were positively correlated (these ratios increase with lignin degradation) and the lignin concentration and the S/V ratio were negatively correlated (these properties decrease with lignin degradation). The C/V ratio was not well represented by the PCA and was expected to be negatively correlated with the lignin degradation axis but is was not the case.

ii. A spot of intact lignin units where the VSC sum are logically positively correlated with its components (V, S and C compounds) (Figure IV-12a).

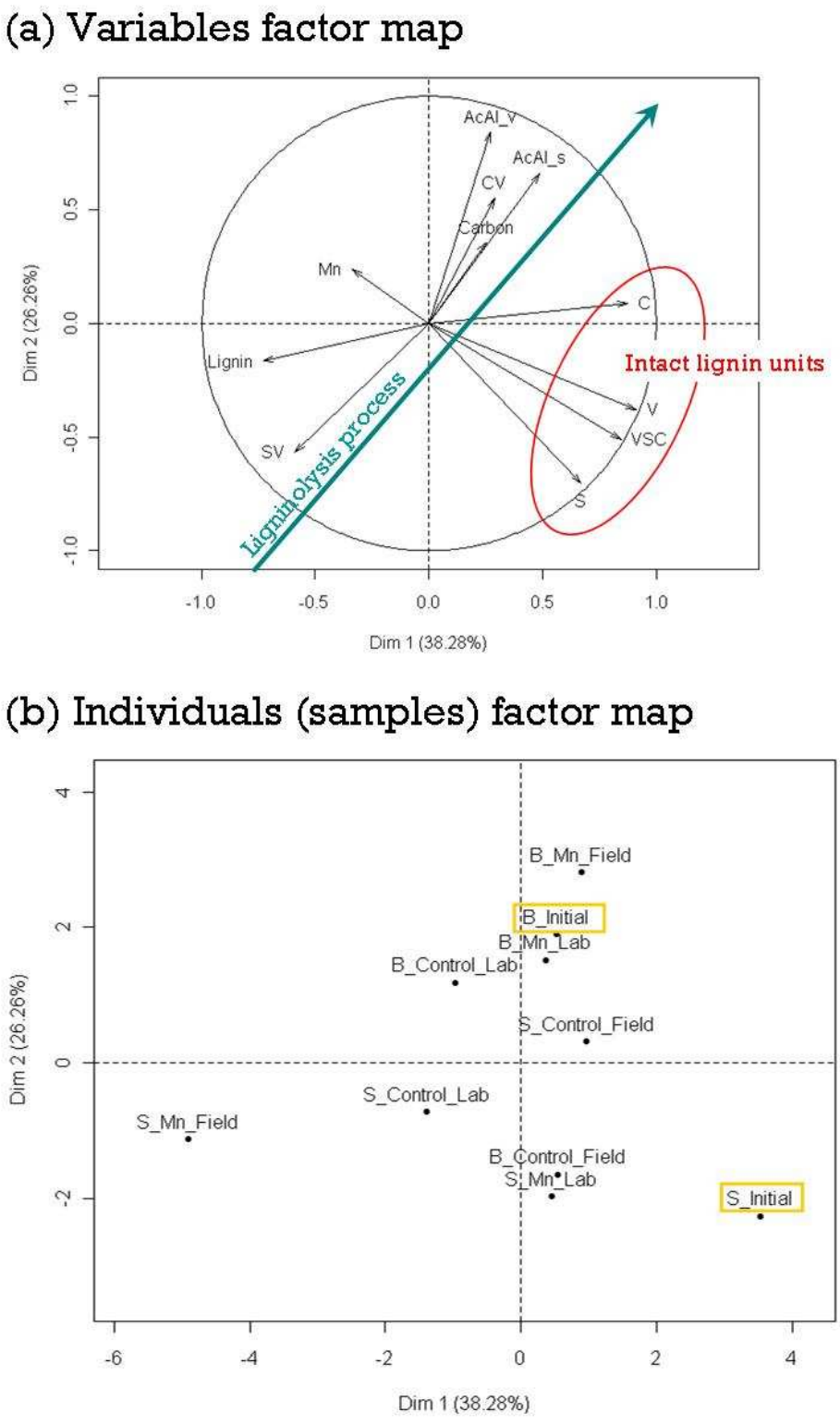


Figure IV-12: The first two dimensions of the PCA performed on our data.

4. Relationships between litter samples

On the basis of PCA results, we performed a clustering analysis to identify possible groups among our samples.

The main findings resulting from this analysis were the following:

(1) The distance of branches on the Figure IV-13 expressed the distance between two clustered elements. Therefore the Mn-enriched branch litters incubated under *in vitro* and *in situ* conditions were the closest among all our samples (Figure IV-13). The reporting of these two samples on the individuals factor map of PCA (Figure IV-12b) showed that they are in the zone of more advanced ligninolysis process.

(2) Control branch and seedling litters *in vitro* incubated constituted a clearly identified cluster (Figure IV-13). These two samples were also represented along the ligninolysis process axes identified by the PCA (Figure IV-12b) but far less than the previous cluster (e.g. Mn-enriched branch litters incubated under *in vitro* and *in situ* conditions).

(3) The two farthest samples were the undecomposed seedling leaves and the *in situ* incubated Mn-enriched seedling litter (Figure IV-13). The reporting of these two samples on the individuals factor map of PCA (Figure IV-12b) showed that the first sample was in the zone of intact lignin units and the second sample was in the opposite direction of this zone.

This clustering did not simply group the samples according to the Mn concentrations, the litter types or the incubation methods. This could be explained by different factors: (i) the Mn concentration in control and Mn-enriched branch litters were in a narrower range of Mn concentration than in seedling litters, (ii) the incubation method (*in vitro vs in situ*) involved differences in terms of temperature and moisture conditions and of microbial communities (the ligninolytic fungi community seem to be absent of the *in vitro* incubation, *cfr* Part IV, Chapter 2).

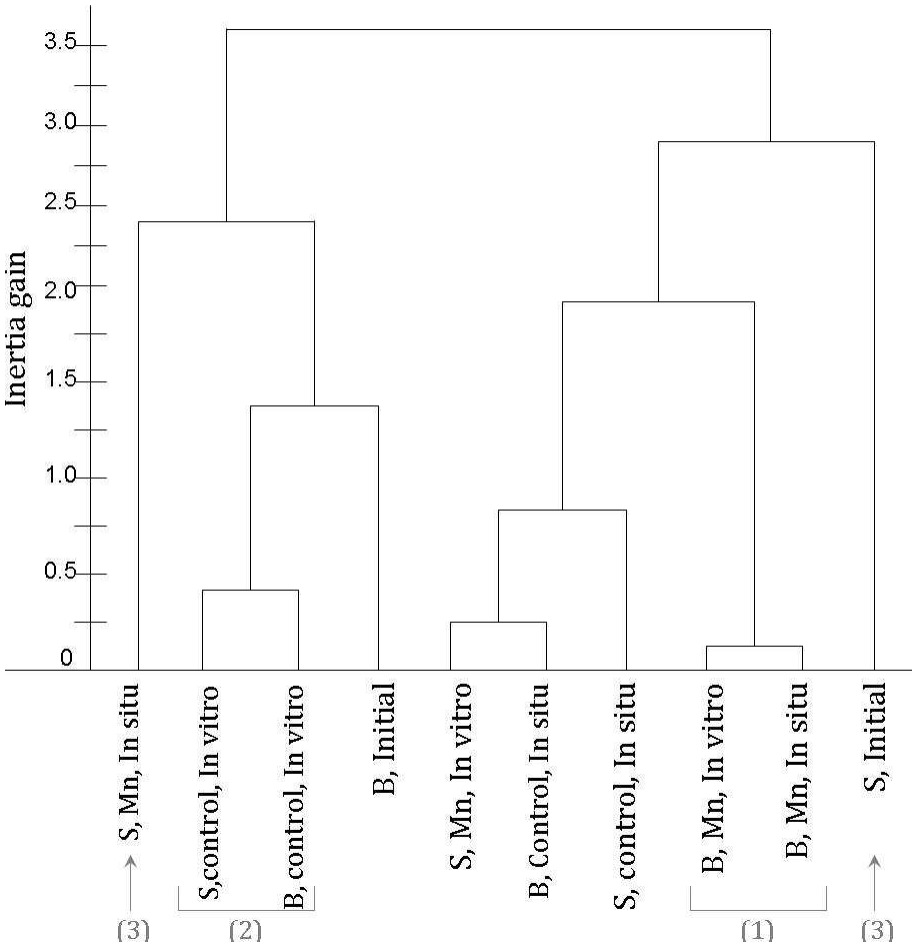


Figure IV-13: Hierarchical clustering of the samples

5. Conclusion

We could not clearly conclude that Mn concentration in litters improve lignin degradation. However, some results support this trend: decrease in VSC sum concentration during *in situ* incubation, increase in increase in $(Ac/Al)_V$ and $(Ac/Al)_S$ ratios in branch litters. This positive influence of Mn is probably mediated by the biotic environment in which the decomposition takes place.

General discussion & Conclusions

After detailed and discussed results of each of our experiments, this last part aims to synthesize these results relative to the initial objective of the thesis. Firstly, we summarize this thesis by giving its leading thread:

Why were we interested in the impact of manganese concentration on forest litter decomposition?

- The lignin degradation may be referred to as *enzymatic combustion* (Kirk and Farell, 1987). In 1984, Kuwara *et al*. discovered the manganese peroxidase (MnP); this enzyme appears to play a key role in lignin degradation and needs Mn^{2+} ion for its catalytic cycle. Perez and Jeffries (1992) also suggested that Mn^{2+} promoted lignin degradation by stabilizing the lignin-degrading enzymes and by enhancing their production.

- Recently, several researchers showed significant positive correlations between the initial Mn concentration and the decay rate (e.g. Berg *et al.*, 2007; Davey *et al.*, 2007; Heim and Frey, 2004, …) . They explained it by the positive influence of Mn on lignin degradation because of its key role in the catalytic cycle of MnP.

What was our specific scientific question?

Those studies are only based on statistical analysis of existing decomposition data from natural ecosystems and do not seem to be unanimous about the exact effect of Mn on lignin degradation.
Therefore, this thesis focused on the impact of Mn concentration on beech (*Fagus sylvaticaI* L.) litter decomposition by controlling the confounding effects.

Which experiments were carried out to answer to this scientific question?

The relationships between the different experiments performed were presented in the "*General introduction & Thesis outline*" (page 8-9) and were schematized in Figure A.

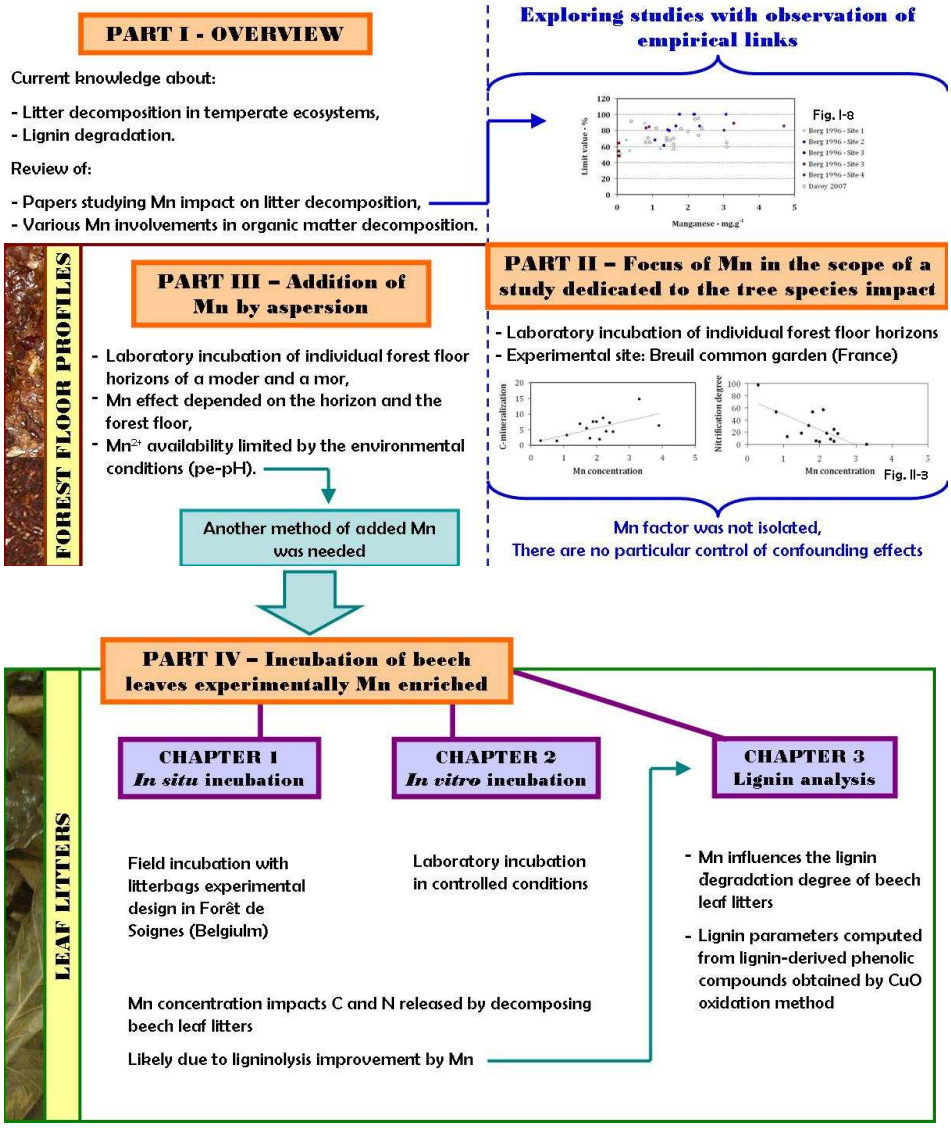


Figure A – Schematic presentation of the thesis outline.

This table (Table A) synthesizes the transversal characteristics of those experiments.

	Experiments described in :				
	Part II	**Part III**	**Part IV**		
			Chapter 1	Chapter 2	Chapter 3
(a) Icubated organic material	Brown litter	Brown litter	Green litter	Green litter	Green litter
(b) Incubation type	Laboratory	Laboratory	Field	Laboratory	Mixte
(c) Temperature condition	4°C	20°C	Depending on the field. $T°_{mean}$ 9.4°C	20°C	Variable
(d) Moisture condition	Moisture as in the sampling field	Field capacity	Depending on the field (mean annual rainfall of 835mm)	Humidity at saturation in the columns	Variable
(e) Different Mn concentrations	Several forest tree species	$MnSO_4$ solution aspersion	Experimental leaves enrichment	Experimental leaves enrichment	Experimental leaves enrichment

What did these differences of experimental characteristics involve?

a) Brown litter *vs* green litter

The brown litter has undergone the process of senescence while the green litter has not. The plant senescence may introduce differences in initial quality of plant litter. The senescence process starts when the leaves are still attached to the plant. This process leads to intensive alteration before the leaves fall down to the ground. Plants withdraw nutrients and other soluble compounds, such as sugars and phenols, before shedding their leaves (Berg and McClaugherty, 2008 in Sanaullah *et al.*, 2010).

Concerning the organic materials studied in this thesis, the green litters exhibited larger nutrient concentrations (Ca, Mg and K) than brown litters (Table B).

Table B – Initial nutrient concentrations in incubated organic materials.

	Brown beech litter			*Green beech litter*	
	Part II	Part III		Part IV	
Litter	Litterfall	Olv (mor)	Olv (moder)	Branch leaves	Seedling leaves
Soil	Cambisol	Podzol	Cambisol	Cambisol	Cambisol
N – %	1.3 ± 0.0	1.9	2.3	2.2 ± 0.2	1.9 ± 0.1
Ca – g/kg	3.2 ± 0.1	8.1	4.2	7.1 ± 0.6	14.2 ± 3.7
Mg – g/kg	0.6 ± 0.1	0.9	0.9	1.4 ± 0.1	2.0 ± 0.2
K – g/kg	3.5 ± 0.1	2.0	1.7	8.4 ± 1.1	8.4 ± 2.5
Na – g/kg	0.1 ± 0.0	0.4	0.1	1.2 ± 0.2	0.1 ± 0.0

b) Field *vs* laboratory incubation

The main difference between field and laboratory incubation concerns the faunal communities involved in the litter decomposition. The field incubation was carried out by using litterbags with an upper mesh size of 2.4 mm allowing access of micro- and meso-fauna (Berg and Laskowski, 2006). In laboratory experiments, only the micro-fauna initially present in the incubated litter sample are involved in decomposition.

c) Temperature and moisture conditions

The temperature and moisture conditions influence the biological activity. Several field studies have shown that temperature is a key factor controlling the rate of decomposition of plant residues. For example, decomposition normally occurs more rapidly in the tropics than in temperate areas. Soil biological activity requires air and moisture. Optimal microbial activity occurs at near field capacity (Linn and Doran, 1984).

The biological activity is measured by the respiration (CO_2 release) (Christ and David, 1996). For example, the release of CO_2 measured in the thesis experiments shows that the CO_2 release was double at 20°C than at 4°C (each litter was beech litter and each soil was a cambisol) (Table C).

Table C – CO_2 release by different incubated organic materials.

Organic material	Study	T°	CO_2 release
Oln	Part II	4°C	0.21 C-mg C-g^{-1} d^{-1}
Olv (moder)	Part III	20°C	0.40 C-mg C-g^{-1} d^{-1}
Branch leaf litter	Part IV / 2	20°C	0.39 – 0.54 C-mg C-g^{-1} d^{-1}
Seedling leaf litter	Part IV / 2	20°C	0.54 – 0.78 C-mg C-g^{-1} d^{-1}

d) Contrasting Mn concentrations

- Forest tree species (Part II)

Litters of different tree species allow a large range of initial Mn concentration. Initial manganese concentration mainly depends on the physiological status of the tree and of the soil pH. In general, there is an opposite relation between pH and the uptake of manganese by plants (Jones, 1957). Litters of different tree species exhibited difference not only in Mn concentration but in whole litter quality.

- Aspersion of $MnSO_4$ solution (Part III)

The disadvantage of this method is that the added Mn^{2+} could evolve under several forms according to the pH and oxidoreduction conditions.

- Leaves experimental enrichment (Part IV)

Litter experimentally enriched in Mn exhibited a similar litter quality except only Mn concentration.

Focus on some results

We observed an impact of Mn in some specific cases. Although significant, the *improvement* of litter decomposition by Mn stayed low. Moreover, our results suggest that the response of litter decomposition to Mn was rather proportional to its concentration than related to the presence of a threshold concentration.

For example:

- In Part III, we observed that carbon mineralization in the Mn treatment was significantly greater by 10 % than in the control treatment of the Olv horizon of mor and moder forest floors.

- In Part IV, chapter 1, we observed a significant positive correlation between the decay constant (*k*) and the initial Mn concentration in litter. The decay rate increases of 0.00005 d^{-1} (about 1/100 of the lowest observed *k*) when the initial Mn concentration in litter increases of 1 $mg.g^{-1}$.

- In Part IV, chapter 2, the cumulative CO_2 release over the incubated period of B-Mn3 branch litters was higher by 25% than that of B-Mn0 branch litter.

The three experiments of Part IV were performed on the same organic materials (litter experimentally Mn-enriched). Then, we performed a PCA analysis with all these results to test if any trends emerge.

All the variables were not well represented by the two first dimensions of this PCA, given that the vectors representing them were rarely close to the correlation circle (Figure B). These two dimensions of the PCA explained 48% of the data.

The first dimension was significantly correlated with:
- DOC concentration in leachates obtained during laboratory experiment (chapter 2), r = 0.91, *P* = 0.0003.
- Specific molar absorptivity of leachates obtained during laboratory experiment (chapter 2), r = 0.90, *P* = 0.0003.
- NO_3^- concentration in leachates obtained during field incubation (chapter 1), r = 0.90, *P* = 0.0003.
- Nitrification degree of leachates obtained during field incubation (chapter 1), r = 0.87, *P* = 0.0010.
- Manganese (Mn^{2+}) concentration in leachates obtained during laboratory experiment (chapter 2), r = -0.81, *P* = 0.0039.
- Specific molar absorptivity of leachates obtained during field experiment (chapter 1), r = -0.85, *P* = 0.0020.

The second dimension was significantly correlated with:
- The S/V ratio obtained by chemolytic analysis of branch and seedling litters (chapter 3), r = 0.91, P = 0.0002.

– The $(Ac/Al)_v$ ratio obtained by chemolytic analysis of branch and seedling litters (chapter 3), r = -0.80, *P* = 0.0053.

Therefore, we interpret the first dimension of the PCA as an axis representing polyphenolic compounds concentration and the second dimension of the PCA as an axis representing ligninolytic process (Figure B). This highlights that the lignin degradation and its consequent release of by-products are key processes to understand the impact of Mn concentration on litter decomposition.

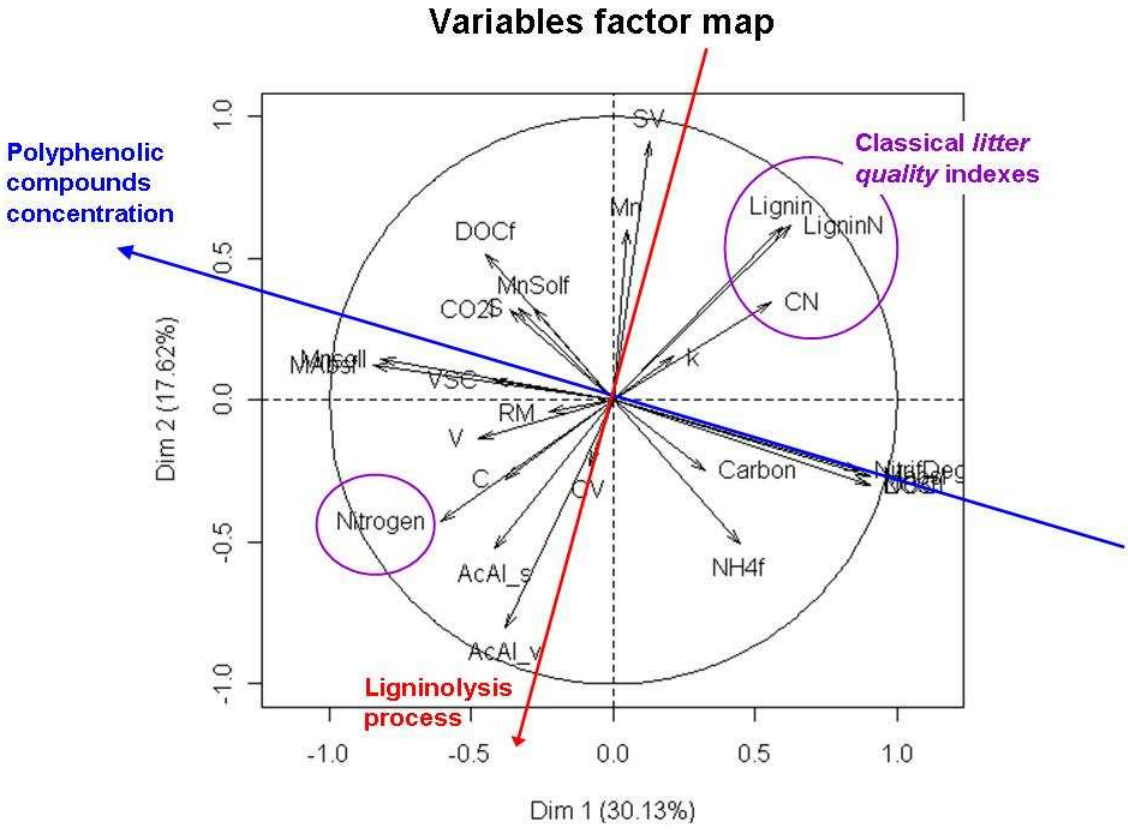


Figure B – PCA, Variable facor map

Perspectives?

On one hand, we propose to focus on the Mn impact at *large* scale by considering the soil, tree and forest floor compartments. Which factors influence the Mn mobility? How do influence the Mn mobility in various conditions (forest tree species and soil types). Do they have a direct or indirect influence on litter decomposition?

On the other hand, we propose to focus on the Mn impact at *small* scale by considering the enzymatic activities and the microbial communities and by monitoring more precisely the lignin alteration degree. What the evolution of lignin alteration indexes during the incubation of litters of contrasting Mn concentrations?

Finally, we think that the role of Mn concentration on N-dynamics in forest floor deserves certainly further attention.

Appendices

APPENDIX 1

Influence of tree species on carbon and nitrogen transformation patterns in forest floor profiles.

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ABSTRACT

Background - Among forest management practices, forest tree species substitution influences biogeochemical cycles and soil interactions rapidly (decades) and significantly.
Methods - We studied the impact of tree species on forest floor properties in 27-year-old monospecific stands of oak, beech, spruce and Douglas-fir, grown in common garden.
Results - In spite of similar litterfall C-inputs (1.3–1.8 Mg ha^{-1} yr^{-1}), C-stocks strongly differed between coniferous (16-21 Mg ha^{-1}) and broadleaved (7-9 Mg ha^{-1}) forest floors. The large C-mineralization under oak (196 C-µg C_{total}-g^{-1} d^{-1}) relatively to other stands (125-147 C-µg C_{total}-g^{-1} d^{-1}) and the large dissolved organic carbon release under broadleaved forest floors may explain this difference. Spruce forest floor exhibited the lowest net nitrification and the largest contribution of organic anions to the anionic charge of aqueous leachate (62.5 % ± 1.7). Conversely, Douglas-fir forest floor exhibited the largest relative nitrification and the largest contribution of nitrate to the anionic charge (70.8 % ± 0.7).
Conclusion - We conclude that tree species induce a specific signature in term of proton donors at the forest floor level (organic *vs* nitric acid), which involves potential impacts on pedogenetic processes in mineral horizon beneath.

KEYWORDS

DOC and CO_2 release - forest floor horizons - N-release - common garden - tree species.

1. INTRODUCTION

The influence of forest tree species on biogeochemical cycles and soil interactions has for a long time attracted numerous scientists and has been covered by an intensive literature. The interest in this research topic originates from the substitution issue of natural vegetation by monospecific coniferous stands as a cause of soil acidification (Binkley and Giardina, 1998). More recently, with the Kyoto protocol, the interest has been rather focused on tree species potential for greenhouse gases mitigation and carbon sequestration (Jandl *et al*., 2007).

Soil acidification and carbon sequestration are influenced by forest floor decomposition through the release of organic/inorganic acids and dissolved organic carbon (DOC) (Guggenberger and Kaiser, 1998). The impact of forest tree species on forest floor properties has been largely studied particularly in common garden. This experimental design makes it possible to minimize confounding effects such as local climate, geology, soil type, forest stand age or past land use management (Binkley and Giardina, 1998; Meentemeyer, 1978) by simultaneous plantations of different tree species in homogeneous pedo-climatic conditions (Gower and Son, 1992; Vesterdal *et al*., 2008). Previous studies in common garden highlighted the following points: (i) tree species can modify topsoil N-mineralization rates in a short time (decades) (Gower and Son, 1992); (ii) forest floors exhibit differences in C/nutrient ratios, pH and nutrient contents according to the tree species (Vesterdal and Raulund-Rasmussen, 1998); (iii) tree species influence microbial and faunal populations through variation in litter lignin and Ca concentration (Hobbie *et al*., 2006); (iv) the influence of tree species on C and N concentration and C/N ratio is more pronounced in the forest floor than in the litterfall and the topsoil because of large differences of turnover rates (Vesterdal *et al*., 2008); (v) Hansen *et al.* (2009) conclude that tree species do not influence the amount of litterfall while site-related factors do and that a large variability in forest floor accumulation could primarily be due to a difference in litter decomposition.

However, the impact of forest tree species on the release of soluble compounds by forest floor has not yet been explored particularly for the Douglas-fir, despite the crucial involvement of these compounds in pedogenetic processes. Moreover, the forest floor is not a *black box*. Forest floor has a heterogeneous composition and can be divided in different layers (horizons) corresponding to successive stages of decomposition (Wardle, 1993). Given that previous studies concluded that the main difference between tree species is decomposition rate (Hansen *et al.*, 2009; Vesterdal *et al.*, 2008), studying forest floor profiles should provide a clearer *picture* of the tree species decomposition process.

The experimental site of Breuil-Chenue in the Morvan mountains (France) is a common garden set up in 1976 specifically to compare biogeochemical cycles between tree species. On this site, no studies have particularly yet considered the forest floor compartment. Nevertheless, Moukoumi *et al*. (2006) has already studied organic matter biodegradability and mineral nutrient availability in the topsoil while Zeller *et al*. (2007) has considered N-transformations in the litterfall and the topsoil.
In this paper, our specific objective is to characterize the release of dissolved products (NH_4^+, NO_3^-, DON, DOC, cations and anions) in forest floor profiles of four tree widespread in Europe: Douglas-fir (*Pseudotsuga menziesii* Franco), Norway spruce (*Picea abies* Karst), European beech (*Fagus sylvatica* L.) and oak (*Quercus sessiliflora* Smith). To support our understanding, we provide a detailed characterisation of the forest floor layers (composition, C- and N-transformation). To achieve this, forest floor horizon samples were collected in the site of Breuil, individually incubated *in vitro* for determination of C- and N-mineralization and leached for measuring the release of soluble compounds.

2. MATERIALS AND METHODS

2.1. Experimental site

The study was carried out on forest floors sampled in the experimental site of Breuil-Chenue set up by the Institut National de la Recherche Agronomique (INRA) in the Natural Park of the Morvan, France (47°18'10" N; 4°4'44" E). The site contains two adjacent blocks with replicated stands. It is established on a plateau at 650 m. Climate is submountain Atlantic, with 1400 mm mean annual rainfall and 6°C mean annual temperature. The native forest was a 150-year-old coppice composed mainly of beech (*Fagus sylvatica* L.) and oak (*Quercus sessiflora* Smith). In 1976, this initial forest was partially clear-felled (one stand was conserved in each block) and replaced by mono-specific 0.1 ha stands of different species (one plot by block) (Bonneau *et al.*, 1977). Previous studies showed that the site heterogeneity was minor (Bonneau *et al.*, 1977; Ranger *et al.*, 2004). Out of the plantations of block 1, we selected the four tree species studied here.
The soil is an acid brown soil, and classified as an Alocrisol (AFES, 1998) or an alumnic Cambisol (IUSS, 2006). It developed from a granite, which was very poor in cation-bearing minerals (0.5% MgO; 0.6% CaO; 4.4% K_2O). The soil is strongly acid, pH is 3.8 in surface horizons and increases with depth (4.7–4.8). Soil texture is sandy-loamy throughout the whole profile, with 40-55% sand and 15-20% clay. Carbon concentrations decrease from 0.44 g kg^{-1} in surface horizons to 0.04 g kg^{-1} in deeper horizons (Bonneau *et al.*, 1977).

2.2. Litterfall and forest floor sampling

Five litter-traps (0.5 m²) were placed in each stand to collect litterfall continuously. Sampling occurred every three months for 3 years between September 2002 and September 2005. Each sample was sorted and dried. All samples of leaf/needle litterfall were considered in order to assess the average annual C-input by litterfall and its variability. For each forest stand, composite samples were analysed for C, N, phosphorus (P), lignin, cellulose and cations concentrations. The composite samples were constituted with leaf/needle litterfall samples from all periods, applying weighting factors according to the dry weight of corresponding litterfall sample.
In each forest stand, we sampled forest floors horizons in December 2003 after leaf fall. We sampled separately Oln (plant remains), Olv (poorly fragmented), Of (fragmented) sub-horizons according to the definitions of Brêthes *et al.* (1995). The material was collected quantitatively on 1m² surface representing forest floor morphology in the plot. An Oh horizon (dark humified organic matter) was collected under spruce, whereas transitional horizons were collected in other stands: Of-Oh under oak and Douglas-fir and Of-Ah under beech. The symbol O^* was used in the following text to identify the deepest horizon of the forest floor which is either Oh or Of-Oh or Of-Ah.

2.3. Analysis of litterfall and forest floor horizons

Carbon and N concentrations were measured by gas chromatography after dry combustion (1800°C) with a Thermo Quest CHN autoanalyzer. We measured the concentration of lignin and cellulose in litterfall by acid-detergent extraction (Van Soest, 1963). Elemental analyses (Ca, Mg, K, Na, Mn, Al and Fe) were carried out by calcination (450°C) and digestion with HNO_3, HF and $HClO_4$ and measured by induced plasma coupled atomic emission spectroscopy (ICP-AES, Jarell Ash). Horizon thicknesses were estimated in the field. Root colonization expressed by the volume percentage taken up by roots in each horizon was visually estimated both in the field and during the sample sorting.

2.4. Incubation, leaching and chemical analysis of leachates

Forest floor materials dedicated to incubation were neither dried nor rewetted before the constitution of columns. Incubation was carried out in PVC-columns with perforated bottom covered by polyamide net (50-µm mesh size). Each column (diameter = 115 mm, height = 200 mm) contained 15 g (equivalent dry weight) of one horizon. All the columns were covered, but not hermetically closed, and pre-incubated during 6 weeks at 4°C. At the end of pre-incubation period, each column was leached with MilliQ water (150 ml).

We introduced two plastic cups in each column: one with 1M NaOH for CO_2 measurement and one with water to balance NaOH-related dehydration. The columns were then closed hermetically. Initial CO_2 content was measured in a closed column without sample (blank column). Columns of separated horizon were incubated in triplicate for 32 days at 4°C. At the end of the incubation period, columns were leached again with MilliQ water (150 ml, with a 1:10 ratio between organic material and water quantity). The leachates were filtered through 0.45-µm pore size Gellman filter and analysed.

We measured pH with CG804 pH-meter, ammonium (NH_4^+) concentration by FIA (Alpkem FS3000), cation concentrations (Na^+, K^+, Ca^{2+}, Mg^{2+}, Al^{3+}, Fe^{2+}, Mn^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+}) by ICP-AES, inorganic anion concentrations (F^-, Cl^-, NO_3^-, NO_2^-, PO_4^{3-}, SO_4^{2-}) by ionic chromatography (HPLC, Dionex LC20 with IONPAC AS 11 column). The anionic deficit charge (ACD) was calculated by the difference between the sum of cationic charge and the sum of anionic charge. The concentration of dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were measured with a total organic carbon analyser (SHIMADZU TV-$V_{CSH-CSN}$). The concentration of dissolved organic nitrogen (DON) was calculated by the difference between TDN and dissolved inorganic nitrogen (DIN), which was defined by the sum of NH_4^+, NO_2^- and NO_3^- concentrations. The release of DOC and DON (C_{DOC}-mg C_{total}-g^{-1} or N_{DON}-mg N_{total}-g^{-1}) was defined by the ratio between the amount of DOC or DON in the leachate after incubation and the initial amount of C or N in the corresponding sample.

2.5. C- and N-mineralization

The production of CO_2 was measured every 10 or 11 days by back titration of NaOH with HCl 1M and deduction of the values from blank columns. C-mineralization was defined by the ratio between the average daily CO_2 production and the initial amount of C in the corresponding sample.
Inorganic N (N-µg N_{total}-g^{-1} d^{-1}) was extracted from 2.5 g (equivalent dry weight) sub-sample from column by using KCl 1M (2.5 g : 100 ml), before and after incubation. NO_3^- and NH_4^+ concentrations in KCl extracts were measured by HPLC (Dionex LC20 with IONPAC AS 11 column) and flow injection analysis (FIA, Alpkem FS3000), respectively.

Net nitrification (N-µg N_{total}-g^{-1} d^{-1}) was defined by the ratio between (1) net NO_3^- production, which is the difference between final and initial NO_3^- concentrations in the KCl extracts, and (2) initial amount of N in the sample. Relative nitrification (%) is defined by the ratio between (1) final NO_3^- in the KCl and water extracts and (2) final mineral N (NH_4^+ and NO_3^-) in the KCl

and water extracts. It is an alternative measure of nitrification considering NO_3^- production in relation to NH_4^+-supplying capacity (Robertson, 1982).
DOC and DON releases, C-mineralization and net nitrification are all defined in function of the initial amount of C or N in the corresponding sample. Using these units facilitates comparison between species since organic matter has different composition between species and horizons.

2.6. Statistical analysis

The differences between means were compared with one-way analysis of variance (ANOVA1) and Fisher's Least Significant Difference (LSD) test, with a significant level of 0.05. All statistical analyses were performed with the SAS software (SAS Institute, 1999). We compared the means between the four tree species, for C- and N-mineralization and for the release of DOC, DON, NH_4^+ and NO_3^- in each horizon. Furthermore, we compared in each forest floor the means of all sub-horizons weighted by their dry mass for C- and N-mineralization and for the release of DOC, DON, NH_4^+ and NO_3^-. We performed as well some correlation analysis between litterfall and forest floor characteristics and C-mineralization with the SAS software.

3. RESULTS

3.1. Forest floor morphology

The forest floor, which encompasses Ol, Of and Oh horizons, was thicker under spruce (40 mm) than under the other tree species (Douglas-fir, 25 mm; beech, 20 mm; oak, 15 mm). The Oh horizon was thick in the spruce forest floor (10-20 mm) while it was thin (< 2 mm) and discontinuous in Douglas-fir and oak forest floors. Oh was absent under beech and we observed mineral particles from Ah horizon in the lower part of Of horizon.

Tree roots colonized Of and Oh horizons whatever the forest floor, but mainly under spruce and beech. They were particularly abundant in the Oh horizon of spruce forest floor where they represented about 25-30 % of the horizon volume. In the other forest floors, we only observed fine roots (1-4 mm) which represented less than 5 % of the volume. Root tips were covered by visible ectomycorrhizal fungi except under Douglas-fir forest floor. The presence of white fungal mats was visually detected in the four forest floors, mainly in Olv.

3.2. Composition of litterfall

The composition of litterfall composite samples is presented in Tab. 1. C-concentrations in litterfall were in the same range of values (47-48 %) under the four species. N-concentrations significantly differed between tree species and were in the order: spruce < oak < beech < Douglas-fir ($P < 0.0001$). P-concentration was the lowest in oak litterfall and the largest in Douglas-fir litterfall ($P < 0.0001$). C:N ratio in litterfall significantly differed between tree species and were in the order: Douglas-fir < beech < oak < spruce ($P < 0.0001$). C:P ratio was the lowest in Douglas-fir litterfall and the largest in oak litterfall ($P < 0.0001$). Lignin concentrations in litterfall were similar under oak, spruce and Douglas-fir (31-34 %) but much larger under beech (54 %, $P = 0.0069$). The concentrations of Ca, Mg and Mn were the largest in Douglas-fir and oak litterfalls and the smallest in spruce litterfall. Beech litterfall exhibited the largest concentrations of K ($P < 0.0001$) and Na ($P < 0.0001$). Douglas-fir litterfall exhibited the largest concentrations of Al ($P < 0.0001$) and Fe ($P = 0.0421$).

Table 1: Composition of the tree species litterfalls. Means with different associated letters are significantly different from one another at the $P < 0.05$ level according to the Fisher's least significant difference test (n=3).

	C	N	P	C/N	C/P	Lignin	Cellulose	Ca	Mg	K	Na	Mn	Al	Fe
	%	%	‰	atomic ratio		%	%	$g.kg^{-1}$						
Oak														
mean	47	1.13	0.45	48	2684	34	26	4.0	0.93	2.3	0.08	2.3	0.13	0.11
SD	1	0.02	0.01	1	63	3	1	0.2	0.05	0.1	0.00	0.1	0.01	0.01
	a	C	c	b	a	b	a	b	a	C	c	b	c	b
Beech														
mean	47	1.33	0.58	42	2095	54	21	3.2	0.58	3.5	0.15	2.0	0.16	0.11
SD	1	0.01	0.01	1	4	8	6	0.1	0.1	0.1	0.01	0.1	0.02	0.01
	a	B	b	c	b	a	a	c	b	A	a	c	c	ab
Spruce														
mean	47	0.98	0.57	55	2128	33	25	3.0	0.54	2.6	0.12	1.7	0.28	0.09
SD	1	0.03	0.03	1	96	4	8	0.2	0.09	0.1	0.01	0.1	0.01	0.01
	a	D	b	a	b	b	a	c	b	B	b	d	b	b
Douglas-fir														
mean	48	1.46	0.72	38	1713	31	25	6.4	0.83	2.4	0.09	2.7	0.54	0.15
SD	1	0.04	0.01	1	22	4	6	0.1	0.08	0.1	0.00	0.1	0.07	0.03
	a	A	a	d	c	b	a	a	a	C	c	a	a	a
P	0.0022	<0.0001	<0.0001	<0.0001	<0.0001	0.0069	0.7692	<0.0001	0.0016	<0.0001	<0.0001	<0.0001	<0.0001	0.0421

3.3. Composition of forest floor horizons

The elemental composition of forest floor horizons is presented in Tab. 2. Whatever the tree species, C-concentration, C:N and C:P ratio decreased with the horizons, N- and P-concentration reached a maximum in Of horizon. The values of C:N ratio in Ol horizons were larger under oak and spruce than under other species. The lowest value of C:P ratio was observed under beech whatever the forest floor horizon. Whatever the tree species, K, Na, Al and Fe concentrations exhibited the same pattern of increase with the horizons. For Mg and Mn concentrations, the differences between species were the largest in Ol horizons.
Weighted average values for whole forest floor (symbolized by the expression *WAV* in the following text) were calculated using the dry weight of each horizon / sub-horizon as weighting factor. The *WAV* of C:N ratio for forest floor were the largest under spruce (32 %) and the smallest under Douglas-fir (24 %). The *WAV* of C:P ratio were the smallest under beech (1221) and the largest under spruce (1875). Beech forest floor presented the largest *WAV* of K, Na, Al and Fe concentrations. Oak forest floor exhibited the largest *WAV* of Ca, Mg and Mn concentrations.

3.4. Composition of forest floor leachates

a. pH, C- and N-release

Douglas-fir leachates strongly decreased in pH with the horizons (Tab. 3). Whatever the forest floor, the greatest DOC release was observed in Olv horizon. The *WAV* of DOC release significantly differed between tree species in the order: Douglas-fir, 1.83 ‰ ± 0.08 < spruce, 2.72 ‰ ± 0.09 < beech, 4.30 ‰ ± 0.07 < oak, 5.96 ‰ ± 0.66; C_{DOC}-mg C_{total}-g^{-1} ($P < 0.0001$). Whatever the forest floor, DON release (N_{DON}-mg N_{total}-g^{-1}) was the largest in Olv horizon leachates, while NH_4^+ release (N-mg N_{total}-g^{-1}) peaked in Of except for beech forest floor (Olv) (Tab. 3). The contribution of N forms (DON, NH_4^+ or NO_3^-) to the total dissolved N-release in leachates changed with the horizons; globally, most of dissolved N was released as DON in upper horizons leachates and as NO_3^- in lower horizons leachates (Fig. 1).
The *WAV* of DON release significantly differed between tree species (Douglas-fir, 0.60 ± 0.44 < spruce, 1.99 ± 0.07 < beech, 2.97 ± 0.35 < oak, 3.75 ± 0.31; N_{DON}-mg N_{total}-g^{-1}; $P < 0.0001$). The *WAV* of NH_4^+ release significantly differed between the broadleaf and coniferous species (oak, 3.90 ± 0.09; beech, 3.91 ± 0.32 > spruce, 2.25 ± 0.09; Douglas-fir, 2.57 ± 0.37; N-mg N_{total}-g^{-1}; $P < 0.0001$). Also, the *WAV* of NO_3^- release significantly differed between tree species (Douglas-fir, 3.10 ± 0.39; oak, 2.78 ± 0.16 > beech, 1.19 ± 0.11 > spruce, 0.24 ± 0.04; N-mg.N_{total}-g^{-1}; $P < 0.0001$).

Table 2: Elemental composition of forest floor horizons and whole forest floor. O* is the deepest horizon of the forest floor which is either Oh, Of-Oh or Of-Ah. The values for whole forest floor were calculated using the dry weight of each horizon / sub-horizon as weighting factor (weighted average values - WAV).

Horizon	species	C	N	P	C/N	C/P	Ca	Mg	K	Na	Mn	Al	Fe
		%	%	‰	atomic ratio		$g.kg^{-1}$	$g.kg^{-1}$	$g.kg^{-1}$	$g.kg^{-1}$	$g.kg^{-1}$	$g.kg^{-1}$	$g.kg^{-1}$
Oln													
	oak	47	1.2	0.46	44	2647	5.5	1.1	2.2	0.1	3.3	1.2	0.5
	beech	50	1.6	0.71	36	1791	3.1	0.5	2.7	0.1	1.5	0.6	0.3
	spruce	48	1.2	0.36	48	3476	3.8	0.3	1.2	0.0	1.7	0.4	0.1
	Douglas-fir	50	1.5	0.47	38	2764	5.5	0.7	1.3	0.1	2.2	0.5	0.2
Olv													
	oak	47	1.4	0.58	38	2103	4.2	0.9	2.3	0.2	2.4	2.0	0.6
	beech	47	2.2	0.83	25	1465	4.0	0.6	2.3	0.3	2.0	2.0	0.6
	spruce	45	1.5	0.58	35	2001	4.3	0.4	1.3	0.1	1.9	1.0	0.3
	Douglas-fir	51	2.5	0.72	24	1817	5.7	0.7	1.4	0.1	2.4	1.5	0.7
Of													
	oak	41	1.9	0.80	25	1312	5.0	0.8	4.2	0.7	3.9	6.7	2.3
	beech	42	2.2	0.93	22	1166	4.1	0.8	4.4	1.2	2.5	11.2	3.1
	spruce	42	1.8	0.70	28	1560	3.2	0.5	3.9	0.7	2.3	6.9	2.1
	Douglas-fir	44	2.3	0.79	22	1435	3.3	0.5	6.3	1.0	1.8	7.9	1.9
O*													
	oak	30	1.5	0.73	23	1063	2.6	1.0	12.5	2.4	2.1	24.8	6.6
	beech	21	1.0	0.62	25	884	1.9	1.0	18.6	4.9	0.8	36.6	8.6
	spruce	33	1.6	0.73	25	1180	1.9	0.7	10.9	2.3	1.1	19.1	4.9
	Douglas-fir	31	1.7	0.64	21	1240	1.3	0.6	10.5	2.3	0.3	16.2	5.6

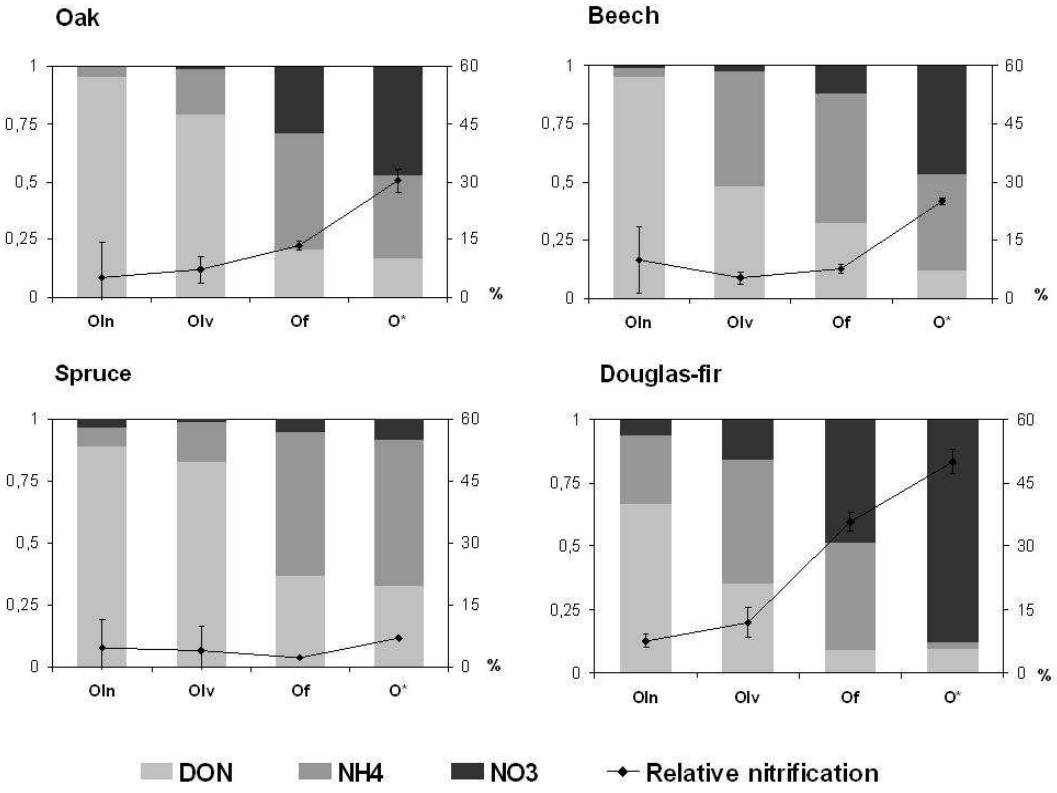
Horizon	species	C	N	P	C/N	C/P	Ca	Mg	K	Na	Mn	Al	Fe
		%	%	‰	atomic ratio		$g.kg^{-1}$	$g.kg^{-1}$	$g.kg^{-1}$	$g.kg^{-1}$	$g.kg^{-1}$	$g.kg^{-1}$	$g.kg^{-1}$
Forest floor													
WAV													
	oak	37	1.6	0.67	27	1435	4.0	0.9	7.2	1.3	2.9	13.1	3.7
	beech	37	1.6	0.77	26	1221	3.1	0.7	8.9	2.2	1.6	17.1	4.2
	spruce	41	1.5	0.57	32	1875	3.1	0.5	5.0	1.0	1.8	8.2	2.2
	Douglas-fir	42	2.1	0.66	24	1628	3.5	0.6	5.9	1.0	1.5	8.2	2.7

Table 3: DOC, DON, NH_4^+ and NO_3^- in forest floor horizon leachates. O^* is the deepest horizon of the forest floor which is either Oh, Of-Oh or Oh-Ah. For each horizon, means with different associated letters are significantly different from one another at the $P < 0.05$ level according to the Fisher's least significant difference test (n=3).

Horizon	**species**	**pH**			**DOC** [C_{DOC}-mg C_{total}-g^{-1}]			**DON** [N_{DON}-mg N_{total}-g^{-1}]			**NH_4^+** [N-mg N_{total}-g^{-1}]			**NO_3^-** [N-mg N_{total}-g^{-1}]		
		mean	SD		mean	SD		mean	SD		mean	SD		mean	SD	
Oln																
	oak	4.0	0.0	d	8.29	1.31	a	5.57	0.74	a	0.28	0.40	ab	0.00	0.00	c
	beech	4.8	0.2	c	3.45	0.53	b	2.35	0.31	b	0.09	0.04	b	0.02	0.04	bc
	spruce	5.3	0.1	b	1.52	0.20	c	2.06	0.20	b	0.18	0.04	b	0.08	0.01	b
	Douglas-fir	6.1	0.1	a	0.78	0.04	c	1.60	0.03	b	0.66	0.12	a	0.15	0.06	a
			P	<0.0001		*P*	<0.0001		*P*	<0.0001		*P*	0.0479		*P*	0.0036
Olv																
	oak	4.4	0.1	c	12.12	3.58	a	9.81	3.16	a	2.44	1.74	b	0.13	0.01	bc
	beech	4.7	0.1	b	8.10	0.60	b	6.12	0.56	b	6.35	0.79	a	0.28	0.12	b
	spruce	4.5	0.0	c	3.09	0.07	c	2.63	0.17	c	0.52	0.09	c	0.03	0.04	c
	Douglas-fir	5.3	0.0	a	2.95	0.31	c	2.01	0.14	c	2.81	0.51	b	0.90	0.20	a
			P	<0.0001		*P*	0.0007		*P*	0.0026		*P*	0.0006		*P*	<0.0001
Of																
	oak	4.7	0.1	a	4.99	0.66	a	3.38	0.64	a	8.21	0.78	a	4.70	0.01	b
	beech	4.6	0.1	ab	4.84	0.37	a	2.77	0.42	ab	4.82	0.63	b	1.03	0.12	c
	spruce	4.5	0.0	b	3.89	0.53	b	2.39	0.44	b	3.78	0.11	b	0.35	0.00	c
	Douglas-fir	4.2	0.1	c	2.54	0.54	c	1.11	0.80	c	5.24	1.77	b	5.97	1.13	a
			P	<0.0001		*P*	0.0002		*P*	0.0087		*P*	0.0038		*P*	<0.0001

Horizon species	**pH**			**DOC** [C_{DOC}-mg C_{total}-g^{-1}]			**DON** [N_{DON}-mg N_{total}-g^{-1}]			**NH_4^+** [N-mg N_{total}-g^{-1}]			**NO_3^-** [N-mg N_{total}-g^{-1}]		
	mean	SD		mean	SD		mean	SD		mean	SD		mean	SD	
O*															
oak	4.4	0.1	a	1.04	0.15	b	1.28	0.16	a	2.70	0.13	a	3.54	0.12	a
beech	4.4	0.1	a	1.35	0.34	ab	0.83	0.03	b	2.89	0.50	a	3.29	0.48	a
spruce	4.4	0.0	a	1.65	0.15	a	1.02	0.14	b	1.84	0.24	b	0.27	0.11	b
Douglas-fir	3.7	0.1	b	0.27	0.07	c	0.38	0.07	c	0.09	0.04	b	3.48	0.68	a
		P	<0.0001		*P*	0.0002		*P*	<0.0001		*P*	<0.0001		*P*	<0.0001

Figure 1: Proportional contributions of DON, NH_4^+ and NO_3^- to total N release in forest floor horizons leachates (left Y-axis) and relative nitrification (%) in forest floor horizons (right Y-axis, mean values are reported ± SD).



b. Anionic signature

The contribution of each anion to the total anionic charge widely differed with the horizons (Fig. 2) and between species (Fig. 3). Whatever the tree species, we observed a strong dominance of organic anions in Ol leachates (65-92 %), except in the leachates of Douglas-fir Olv (38 % ± 10). Most of anions was released as NO_3^- in Of and O^* leachates of oak, beech and Douglas-fir (65-85 %).

In the whole forest floor (*WAV*) (Fig. 3), NO_3^- strongly dominated in Douglas-fir leachates (71 % ± 1) while it was organic anions in spruce leachates (62 % ± 2). In oak and beech leachates, both NO_3^- and organic anions contributed largely to the anionic charge, but the contribution of NO_3^- was larger for oak than for beech (41 % ± 4 *vs* 30 % ± 3; $P < 0.0001$).

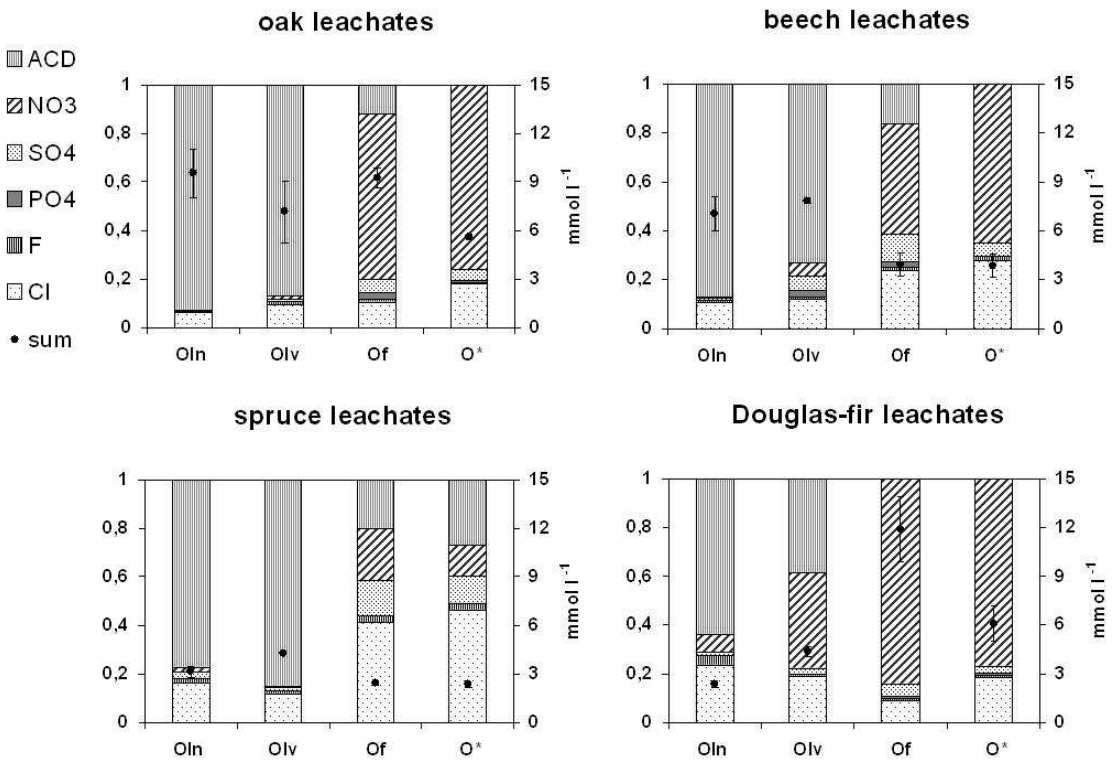


Figure 2: Proportional contributions of anions (ACD^*, NO_3^-, SO_4^{2-}, PO_4^{3-}, F^- and Cl^-) in forest floor horizons leachates (left Y-axis) and sum of anions in $mmol.l^{-1}$ (right Y-axis, mean values are reported ± SD). (*) ACD is the "deficit in anionic charge".

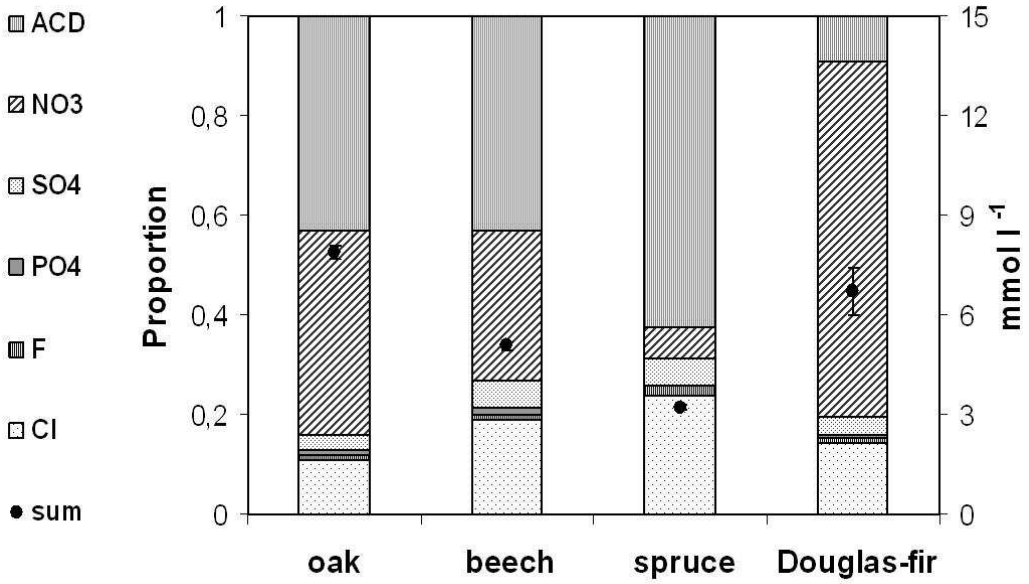


Figure 3: Proportional contributions of anions (ACD, NO_3^-, SO_4^{2-}, PO_4^{3-}, F^- and Cl^-) in the whole forest floors leachates (left Y-axis) and sum of anions in mmol.l^{-1} (right Y-axis, mean values are reported ± SD). The values for the whole forest floor were calculated using the dry weight of each horizon / sub-horizon as weighting factor (weighted average values - *WAV*).

3.5. C- and N-mineralization in forest floor horizons and whole forest floors

Whatever the tree species, C-mineralization (C-µg C_{total}-g^{-1} d^{-1}) strongly decreased from Ol to O* (Fig. 4). The *WAV* of C-mineralization computed for each forest floor (Tab. 4) was the largest in the oak forest floor ($P < 0.0001$). By contrast, we observed only minor differences of C-mineralization between beech, spruce and Douglas-fir forest floors.
Whatever the tree species, the relative nitrification (%) increased from Of to O* (Fig. 5). However, this increase was very low under spruce in comparison with the other species. In Oln, relative nitrification (%) did not significantly differ between tree species (Tab. 4). In Olv, Of and O*, the greatest values of relative nitrification were observed under Douglas-fir whereas the smallest values were observed under spruce (Olv, $P = 0.1386$; Of, $P < 0.0001$; O*, $P < 0.0001$). The *WAV* of relative nitrification significantly decreased in the following order: Douglas-fir > oak > beech > spruce ($P < 0.0001$).

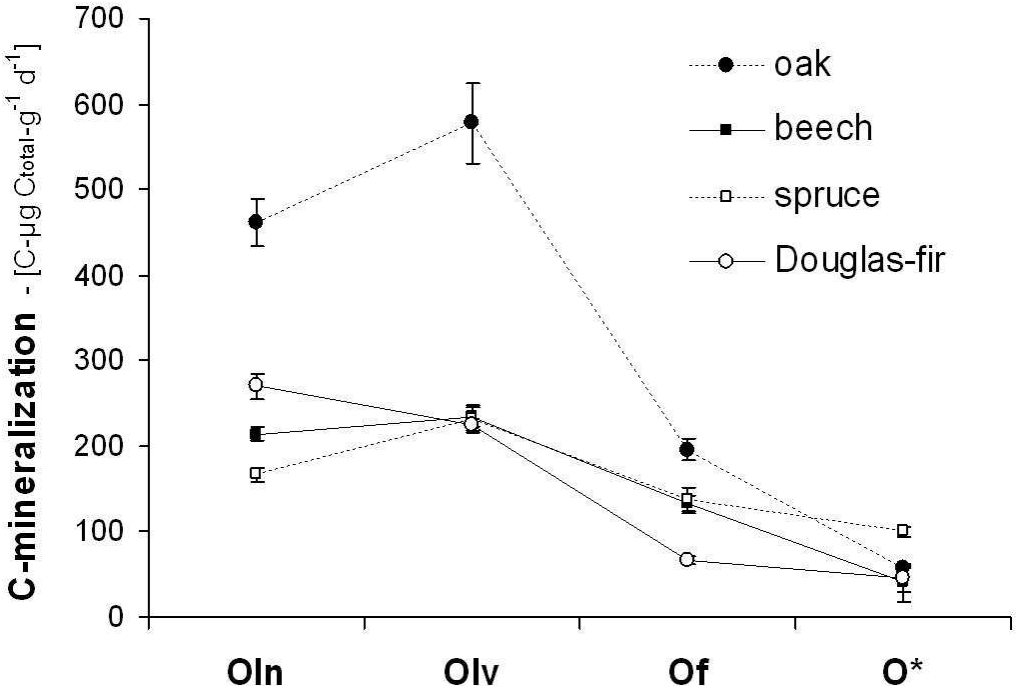


Figure 4: C-mineralization in forest floor horizons of each species.

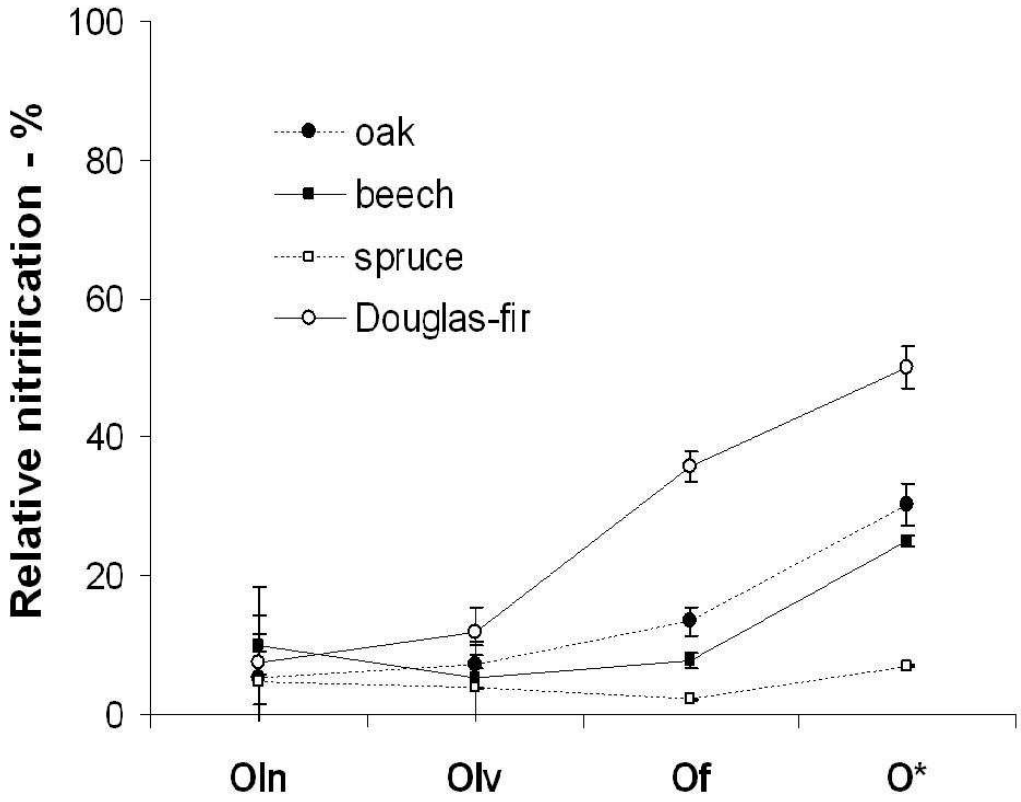


Figure 5: Relative nitrification in forest floor horizons of each species.

Table 4: C-mineralization, net N-nitrification and relative nitrification in forest floor horizons and in the whole forest floor.
The relative nitrification (%) is defined by the ratio between (1) final NO_3^- in the KCl and water extracts and (2) final mineral N (NH_4^+ and NO_3^-) in the KCl and water extracts. The values for the whole forest floor were calculated using the dry weight of each horizon / sub-horizon as weighting factor (weighted average values - *WAV*). For each horizon, means with different associated letters are significantly different from one another at the $P < 0.05$ level according to the Fisher's least significant difference test (n=3).

horizon	species	Carbon mineralization [C-µg C_{total}-g^{-1} d^{-1}]			N-nitrification [N-µg N_{total}-g^{-1} d^{-1}]			Relative nitrification %		
		mean	SD		mean	SD		mean	SD	
Oln										
	oak	462	28	a	15	26	a	5	9	a
	beech	214	8	c	32	28	a	10	9	a
	spruce	167	8	d	1	1	a	5	7	a
	Douglas-fir	270	15	b	37	11	a	7	2	a
			P	<0.0001		*P* =	0.1825		*P* =	0.8006
Olv										
	oak	578	47	a	60	5	a	7	3	ab
	beech	234	12	b	35	6	b	5	1	ab
	spruce	232	14	b	9	14	c	4	6	b
	Douglas-fir	224	7	b	69	8	a	12	3	a
			P	<0.0001		*P* =	0.0002		*P* =	0.1386
Of										
	oak	196	13	a	185	31	a	13	2	b
	beech	133	10	b	34	10	c	8	1	c
	spruce	138	13	b	19	3	c	2	0	d
	Douglas-fir	67	4	c	110	26	b	36	2	a
			P	<0.0001		*P*	<0.0001		*P*	<0.0001
O*										
	oak	57	0	b	122	24	a	30	3	b
	beech	40	21	b	73	21	ab	25	1	c
	spruce	100	5	a	34	3	b	7	0	d
	Douglas-fir	45	15	b	96	44	a	50	3	a
			P =	0.0021		*P* =	0.0232		*P*	<0.0001
Forest floor - WAV										
	oak	196	9	a	138	22	a	19	2	b
	beech	130	10	c	42	4	c	11	1	c
	spruce	147	3	b	19	3	d	4	1	d
	Douglas-fir	125	6	c	86	20	b	30	1	a
			P	<0.0001		*P*	<0.0001		*P*	<0.0001

4. DISCUSSION

Litter decomposition is mainly affected by climate, soil and vegetation (Meentemeyer, 1978). Here, the four forest tree stands developed in homogeneous conditions of soil and climate (Ranger *et al.*, 2004). The differences of forest floor properties are closely linked with tree species.

4.1. Impact of tree species on litterfall quality

As observed by Alban (1982), litterfall composition is significantly affected by tree species and might directly affect the composition of the whole forest floor. The concentrations of K, Na and Mg in the forest floor were positively correlated to K, Na and Mg concentrations in litterfall (r = 0.71, 0.72 and 0.69, respectively). There was also a strong correlation (r = 0.97, $P = 0.0368$, n = 4) between C:N in forest floors and corresponding litterfalls. However, Vesterdal *et al.* (2008) studied 6 tree species including all of ours except Douglas-fir in a common garden and found no correlation between C:N of forest floors and corresponding litterfalls. The particularly low C:N in Douglas-fir forest floor and litterfall could explain this divergence. Vesterdal and Raulund-Rasmussen (1998) found also C:N ratio in Douglas-fir forest floor significantly lower than in 6 other tree species forest floors in average on 7 sites of different nutrient status.

Vesterdal and Raulund-Rasmussen (1998) concluded that differences in forest floor nutrient status may not only reflect differences in litter nutrient status and that the initial nutrient status of litter has probably been mediated by variable decomposition rates and nutrient dynamics.
In our study, the concentration of Ca, Mn, Al and Fe in forest floors were poorly correlated to corresponding concentrations in litterfall. This absence of clear relationship could be attributed to differences between species concerning (i) the loss of these cations from forest floor horizons by leaching and root uptake (Currie *et al.*, 1999) and/or (ii) the relative accumulation of divalent cations and metals as a result of C-loss by mineralization. These cations are strongly bound to humic compounds, as compared to monovalent cations and Mg (Yavitt and Fahey, 1984). The evolution of cation concentrations during litter decay should be influenced by the balance between cation- and C-release. If we apply these hypotheses to our data, the higher C-mineralization under oak as compared with other species could partly explain the highest concentrations of Ca, Mg and Mn in oak forest floor. Vesterdal (1998) found also that oak tended to have higher basal respiration rate than the other species. Furthermore, spruce roots preferentially colonize forest floor (Puhe, 2003). Root uptake in this forest floor may reduce cation release.

4.2. Impact of tree species on C- and N-transformation

As previously reported, our results support that (i) tree species exert a strong control on C- and N-cycling (Gower and Son, 1992; Lovett *et al.*, 2004), particularly on nitrification (Zeller *et al.*, 2007), (ii) tree species can modify N-cycling rates in a short time (decades) relative to soil development (Gower and Son, 1992). In addition, our study shows that the profile pattern of C- and N-transformation was not impacted by the tree species and that only the "intensity" of C- and N-transformation was impacted.

Our results are consistent with previous studies on the same experimental site. The effect of tree species on C-mineralization in forest floors was identical to their effect on C-mineralization in Ah horizon measured by Moukoumi *et al.* (2006) (spruce > beech > Douglas-fir, oak was not considered in their study). As often reported in the literature, beneath the spruce stand, a very low value of nitrification was observed both in Ah horizon (Zeller *et al.*, 2007) and in forest floor. It might be attributed to the relatively large C:N in litterfall. Nitrification might be affected by roots, either through the competitive absorption of NH_4^+ (Hart *et al.*, 1994) or through the excretion of molecules affecting microorganisms (Turpault *et al.*, 2007). Living roots were not included in our experiments, but the composition of microbial populations may be impacted by contact with roots before sampling.

In our study, the large rates of C-mineralization in oak forest floor, relatively to other tree species can not be explained by littterfall composition. No correlation was observed between C-mineralization and litter quality indexes as C:N, lignin:N and lignin defined by Melillo and Aber (1982) or as Mn concentration defined by Berg *et al.* (2007). This could be primarily attributed to discrepancy in litter decomposition. Such discrepancy could come from absence of allelopathic compounds (Kraus *et al.*, 2003; Lorenz *et al.*, 2004). Kuiters and Denneman (1987) reported that polyphenol concentrations were higher in the forest floor and soil under Norway spruce and beech than under oak. It could also come from difference in micro- and meso-organism populations (Hobbie *et al.*, 2006; Templer *et al.*, 2003) and from different microbial access to organic N and P sources rather than by different total amounts of N and P (Vesterdal, 1998).

The contribution of N forms (DON, NH_4^+ and NO_3^-) in leachates changes according to the forest floor horizons. Our results confirmed the suggestion of Qualls *et al.* (1991): DON leached from the forest floor originates essentially from the upper forest floor where soluble organic matters are present. In lower horizons leachates (Of and O^*), DIN (= NH_4^+ and NO_3^-) dominated, corresponding to larger net ammonification and net nitrification.

4.3. Impact of tree species on C- turnover

C-stocks in forest floor were smallest under broaleaved species (oak: 9.1 Mg ha^{-1} ± 3.2; beech: 7.8 Mg ha^{-1} ± 2.6 < spruce: 21.5 Mg ha^{-1} ± 11.6; Douglas-fir: 16.4 Mg ha^{-1} ± 4.9, data from Ranger *et al.*, 2004), although C-input from litterfall was similar whatever the tree species, which probably reveals a faster C-turnover in oak and beech forest floors as compared with coniferous species. This faster C-turnover might be related to (i) the larger C-mineralization in oak forest floor and (ii) the larger DOC releases from both oak and beech forest floors. Our results showed that DOC release must be considered in C-turnover calculation, because it accounted for 22% (d), 40% (b), 51% (c) and 56% (d) ($P < 0.0001$) of the total C-output (CO_2 + DOC), under Douglas-fir, spruce, beech and oak, respectively. Such large DOC contribution to C-output was also observed in the studies of Qualls *et al.* (1991) and Yavitt and Fahey (1984) which reported that about 30% of the annual C-release (CO_2 + DOC) from a lodgepole pine forest floor was DOC. Fröberg *et al.* (2007) even observed that DOC loss accounted for 42% of the total mass loss of a litter of Norway spruce.

4.4. Impact of tree species on the release of aqueous proton donors

The anionic signature widely differed between the four tree species. The strongest contrast was observed between spruce and Douglas-fir, with dominance of NO_3^- in Douglas-fir leachates, and organic anions in spruce leachates. According to Cronan (1978), the anionic charge deficit (ACD) may be largely attributed to deprotonated organic acids. The larger NO_3^- contribution in Douglas-fir leachates can be attributed to the larger net nitrification and low DOC release in this forest floor as compared with other species. A high value of relative nitrification in Douglas-fir forest floor may explain the low pH values in corresponding leachates. Indeed, relative nitrification might be considered as a balance between nitrification and ammonification, which directly affects proton budget in soils, through either proton release or proton consumption (Van Breemen *et al.*, 1983).

The composition of forest floor leachates has direct impact on soil development. Nitric and organic acids induce mineral weathering (Ugolini and Sletten, 1991), whereas corresponding anions may contribute to the leaching of cations and metals from topsoil, leading to nutrient depletion, soil acidification and podzolization (Aber, 1992; Currie *et al.*, 1999; Van Breemen *et al.*, 1983). Low pH under Douglas-fir and oak may promote mineral weathering, while large DOC concentration under spruce may promote acido-complexolysis (Augusto *et al.*, 1998).

4.5. Conclusion

We conclude that tree species induces a specific signature of proton donors at the forest floor level (organic *vs* nitric acid) because of their impact on C- and N-cycling. The release of proton donors can directly impact pedogenetic process in mineral horizons beneath. Consequently, soil degradation could be influenced by setting up of well-chosen tree species stand. However, as root uptake of cations and anions is not considered in our experiment, we cannot draw further conclusions on the effect of root uptake of nutrients on the control of nitrification. We believe that interesting perspectives could involve *in situ* studies taking into account key processes impacted by tree species: nutrient uptake, root exudation, element leaching from canopy.

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APPENDIX 2

Experimental methods determining lignin quantity and quality

Determination of biochemical composition by Van Soest method

This method fractionates biomass fibre by successive chemical extractions and quantifies by gravimetry dry insoluble fibre obtained after extraction. These different extraction and quantification steps are presented in Figure A-1.

Method steps:

1) **Ash content** was carried out by sample calcination during 24 h at 450°C.

2) **Lignin and cellulose content** were obtained after solubilisation in acid detergent.

- On litre of acid detergent is constituted of sulphuric acid (1N) with 20 g of cethyltrimethylammonium bromide, 5 g of sodium sulphate and some ml of decahydronaphthalene.
- The dry samples (1 g) are solubilised in acid detergent solution during 1 h at 100°C.
- The extracted samples are washed three times in hot water and three times in acetone.
- Then, the extracted samples are dried during 24 h at 100°C. The ratio between the extracted sample mass (g) and the initial sample mass (g) represents the contents (%) of lignin and cellulose in the initial sample.

3) **Lignin content** was obtained after solubilisation in sulphuric acid

- The dry samples (already solubilised in acid detergent) are extracted in sulphuric acid (72 %) three times during 1 h.
- They are washed three times in hot water.
- Then, the extracted samples are dried during 24 h at 100°C. The ratio between the extracted sample mass (g) and the initial sample mass (g) represents the contents (%) of lignin in the initial sample.

Remarks:

This method is also widely used, especially for analysis of cereals, forages and feed ingredients which often have high contents of proteins and other non lignocellulosic components (Kirk, 1998). The major disadvantages of this procedure are as follows:

- Other components, including proteins and suberins, may condense and analyse as lignin.
- Some lignins, notably those rich in syringyl residues (angiosperm tissues) are potentially solubilised.
- For decomposing organic materials, the term "lignin" includes intact and partially degraded lignins (Berg, 1986).

Reference: Van Soest P.J., 1963, Use of detergent in the analysis of fibrous feeds. II. A rapid method for the determination of fibre and lignin, J. Ass. Office. Anal. Chem, 46: 829-835.

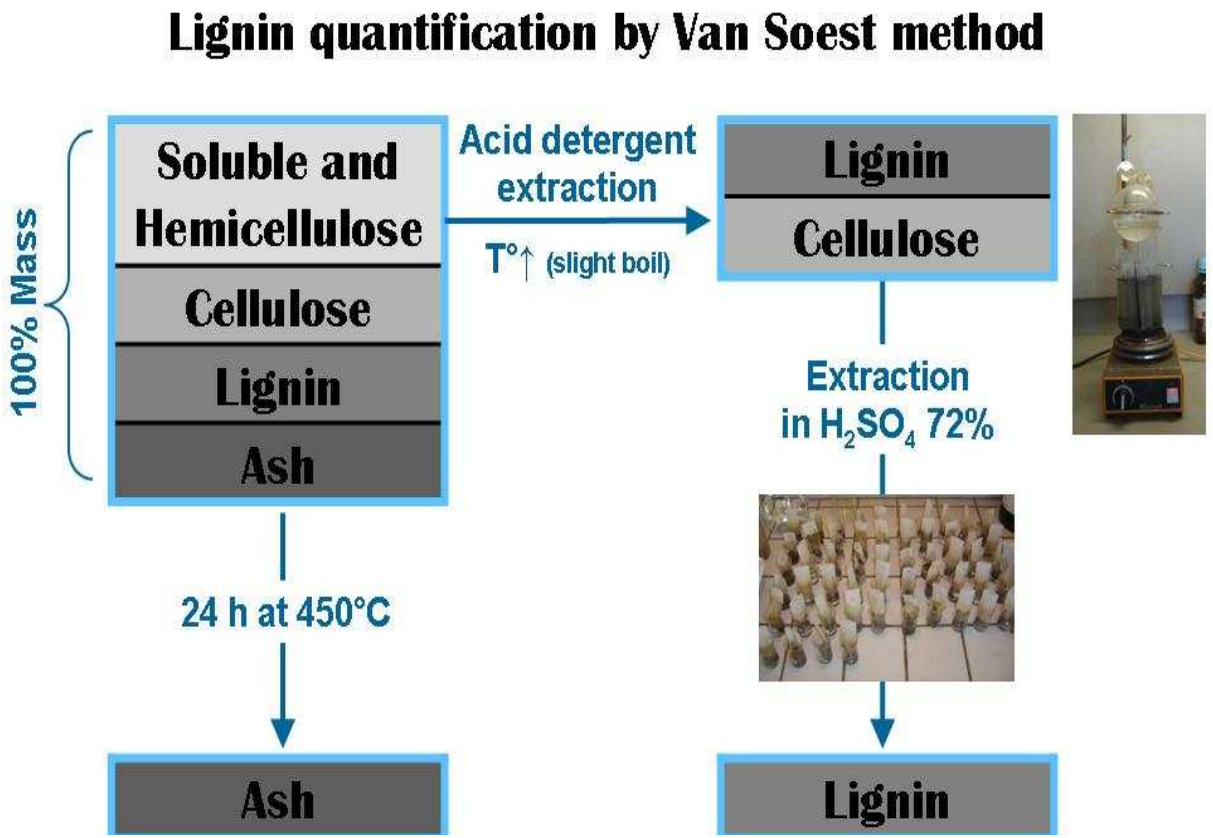

Figure A-1: Flow diagram for forage fibre determination of proximate carbon fractions (modified from Ryan, 1990).

Determination of lignin-derived phenolic compounds by CuO oxidation

One of the standard procedures used to investigate the composition of lignins involves the use of alkaline CuO as a weak oxidant to isolate the constituent phenylpropanoid moieties (PPDs) which they can be determined by HPLC. The CuO oxidation is a relatively mild oxidation yielding primarily simple phenolics whereas condensed lignin structures are not attacked. Treatment of gymnosperm wood produces guaiacyl-type phenols (**vanillin and vanillic acid**), angiosperm wood both guaiacyl- and syringyl-type phenols (**syringaldehyde and syringic acid**). Non woody tissues give additionally cinnamyl phenols (***p*-coumaric and ferulic acid**).

The sum of these phenolics products calls VSC:

- The vanillyl compounds (V) are vanillic acid and vanillin,
- The syringyl compounds (S) are syringic acid and syringaldehyde,
- The cinnamyl compounds (C) are p-coumaric acid and ferulic acid.

These six products are used to estimate (i) the lignin content and (ii) de lignin degradation state.

Method steps:

1) Samples are dried (at 40° C) and ground (< 0.75 mm).

2) **Alkaline CuO oxidation digestion** (performed in EFOR laboratory)

- An equivalent of 50 mg dry mass of samples was oxidized in *teflon vials* with
  - 250 ± 5 mg of powdered CuO,
  - 50 ± 5 mg de $Fe(NH_4)_2(SO_4)_2.6H_2O$,
  - 10 ml of NaOH (2M).
- The oxidation was carried out under N2 atmosphere for 2.5 h at 170°C.

3) After cooling, **oxidation products were purified** using a combination of washing, centrifugation and acidification (with HCl 6M).

- The suspension is centrifuged with 10 ml of demineralised water for 25 min at 3000 rev/min.
- The supernatant is saved and the sediments are centrifugated as before.
- The combined supernatants are acidifies to pH 2 with HCl (6 M) and held at room temperature for 1 h.
- The suspension is included in a volume of 50 ml with demineralised water.

4) **Analysis of the oxidation products** (performed in INBR laboratory)

- The separation and the quantification of the oxidation products were carried out by HPLC .
- HPLC system:

  Waters 1525 Binary HPLC pump
  Rheodyne 5 µL injector
  Waters Symmetry C18 3.5 µm HPLC column 4.6 X 75 mm
  Waters 2489 UV/Vis detector
  Waters Breeze 2 acquisition software

- Chromatographic conditions:

  Isocratic eluant: 850/152/5 (% vol)
  H2O/MeOH/H3PO4
  25 °C - 0.7 ml/min

Reference: (Hedges and Ertel, 1982) as modified by (Kögel and Bochter, 1985).

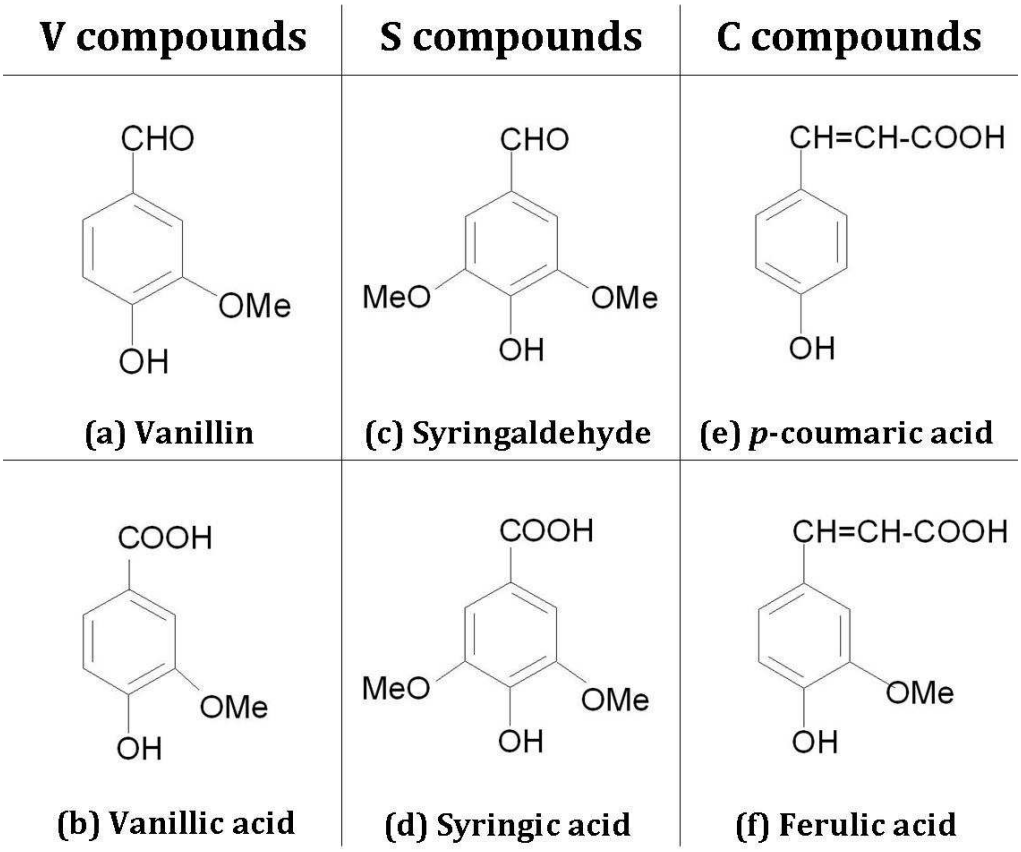

Figure A-2: Determined products from alkaline CuO oxidation of lignin (from Kögel, 1986).

List of relate communications

Papers

Trum F., Titeux H., Ranger J. and Delvaux B., **2011**. *Influence of tree species on carbon and nitrogen transformation patterns in forest floors profiles.* Annals of Forest Science, DOI 10.1007/s13595-011-0080-4.

Trum F., Titeux H., Cornelis J-T. and Delvaux B., **2011**. *Effects of manganese addition on carbon release from forest floor horizons*. Canadian Journal of Forest Research 41: 643-648.

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Participation in conferences

07/2011 **SOM2011** – International symposium on soil organic matter, Leuven (Belgium).
Trum F, Titeux H and Delvaux B: *Manganese concentration, an indicator of carbon sequestration? – Field study on the influence of manganese on beech leaf litters decomposition.* (poster)

06/2011 **ISMOM2011** - 6th International Symposium of Interactions of Soil Minerals with Organic Components and Microorganisms, Montpellier (France).
Trum F and Delvaux B: *Manganese effect on lignin degradation degree in beech (Fagus sylvatica L.) leaf litters*. (poster)

09/2010 **SOM2010** - Organic matter stabilization and ecosystem function, Prequ'île de Giens (France).
Trum F, Titeux H and Delvaux B: *Influence of manganese concentration in beech leaf litter on lignin quality, carbon (DOC) and nitrogen releases during decomposition - field experiment.* (poster)

08/2010 **WCSS2010** - 19th world congress of soil science, Brisbane (Australia).
Trum F, Titeux H and Delvaux B: *Effects of Mn concentration on beech leaf litter decomposition - Results from field and laboratory experiments.* (poster)

09/2009 **ISEB2009** - International symposium on environmental biogeochemistry, Hamburg (Germany).
Trum F, Titeux H and Delvaux B: *Effect of Mn concentration in beech leaf litter on CO_2, DOC, NH_4 and NO_3 releases during incubation.* (oral presentation)

07/2009 **BIOGEOMON2009** – 6th symposium on ecosystem behaviour, Helsinki (Finland).
Trum F, Titeux H and Delvaux B: *Effect of Mn concentration in beech leaf litter on CO_2 and DOC releases during incubation.* (poster)

03/2009 Attendance to **SOM summerschool** "Soil organic matter – composition and turnover", Freising (Germany).
Trum F: *Influence of Mn on forest floors decomposition in temperate ecosystems.* (poster)

08/2008 **EUROSOIL2008**, Vienna (Austria).
Trum F, Titeux H, Ranger J and Delvaux B: *Impact of tree species on the release from forest floor of organic and inorganic carbon and nitrogen.* (oral presentation)

08/2008 **EUROSOIL2008**, Vienna (Austria).
Trum F, Titeux H and Delvaux B : *Impact of Mn^2 addition release (DOC and CO_2) from forest floor horizons.* (poster)

02/2008 **Day of young scientists of BSSS** (Belgian soil society of soil science), Brussels.
Trum F, Titeux H and Delvaux B: *Impact of Mn^2 addition release (DOC and CO_2) from forest floor horizons.* (oral presentation)

01/2008 **2nd ENVITAM PhD day**, Liège.
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02/2005 **Day of young scientists of BSSS** (Belgian soil society of soil science), Brussels.
Trum F, Titeux H, Ranger J and Delvaux B: *Impact of forest floor tree species on the chemical composition of forest floor leachate.* (oral presentation)

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