Anoxic conditions in a Douglas fir litter layer

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1 Introduction

1.1 General introduction

Scope

Anoxic conditions in soils give rise to anaerobic microbial processes, such as denitrification. Anaerobic processes have received considerable attention during the last decades as they play an important role in the production of greenhouse gases, such as N\textsubscript{2}O (Delwiche, 1981; Conrad, 1996). Nitrous oxide is also important for the atmospheric ozone content (Crutzen, 1970). The global production of N\textsubscript{2}O is increasing, for a considerable part due to the grown application of N fertilizers in modern agriculture (Duxbury et al., 1993). Increased surface application of manure has led to regional atmospheric N deposition in forests, far higher than natural background deposition (Van Breemen and Verstraten, 1991). Atmospheric N deposition has affected nitrogen transformation processes in forests, leading to high rates of nitrogen mineralisation and nitrification (McNulty et al., 1990; Tietema et al., 1992a,b). Enhanced nitrification has led to acidification (Van Breemen et al., 1982; Tietema and Verstraten, 1992) and increased nitrous oxide production by nitrifiers as well as denitrifiers (Martikainen, 1985; Martikainen et al., 1993; Sitaula and Bakken, 1993; Sitaula et al., 1995; Butterbach-Bahl et al., 1997). N\textsubscript{2}O production rates in temperate forest soils range from 0.02-20 kg N\textsubscript{2}O-N ha\textsuperscript{-1} y\textsuperscript{-1} (Papen and Butterbach-Bahl, 1999).

An area with high atmospheric deposition and availability of nitrogen is the Veluwe, which is in the central part of The Netherlands (Tietema et al., 1992a,b). In this area forests are mainly located on well-drained sandy soils. In these forests, nitrification and N mineralisation rates are considerable and in general are highest in the litter layer (Tietema et al., 1992a; Koopmans et al., 1995). Denitrification studies that separate between denitrification in the litter layer and in the mineral soil, show that denitrification or N\textsubscript{2}O production in the litter layer was equally or even more important (up to 70-80\%) than denitrification or N\textsubscript{2}O production in the mineral soil (Martikainen et al., 1993; Musacchio et al., 1996; Dong et al., 1998; Regina et al., 1998; Papen and Butterbach-Bahl, 1999). This leads to the question when and where anoxic conditions occur in a forest floor. In general, it is thought that the high porosity of forest soils does not allow for the development of anoxic conditions. But on the other hand, the water holding capacity of forest floors is high and may lead to a decreased diffusivity of oxygen at times of intense rainfall (Freijer, 1994a).

Knowledge of the occurrence of anoxic conditions and denitrification in well-drained temperate forest soils, is important, as such forests cover a considerable area and may be of significance in the global N\textsubscript{2}O budget (Papen and Butterbach-Bahl, 1999). In addition, denitrification might also be important in the consumption of protons in acidified forest soils (Van Breemen and Verstraten, 1991).
Anoxic conditions in a Douglas fir litter layer and their relation with denitrification are the topics of this thesis.

**Biological denitrification**

Biological denitrification is defined as the microbial reduction of nitrogen oxides to nitric oxide, nitrous oxide and nitrogen gas (Knowles, 1981). Denitrification in soil has been thought to occur mainly under anoxic conditions, although denitrification has been found to occur in the presence of oxygen in laboratory studies with pure cultures (Carter et al., 1995; Robertson et al., 1995; Otte et al., 1996). The biochemical pathway of denitrification is (Knowles, 1981):

\[
\begin{align*}
\text{NaR} & \quad \text{NiR} & \quad \text{NOR} & \quad \text{NOS} \\
\text{NO}_3^- & \Rightarrow \text{NO}_2^- & \Rightarrow \text{NO} & \Rightarrow \text{N}_2\text{O} & \Rightarrow \text{N}_2
\end{align*}
\]

The reactions in this pathway are catalysed by the enzymes nitrate reductase (NaR), nitrite reductase (NiR), nitric oxide reductase (NOR) and nitrous oxide reductase (NOS). The activity and synthesis of these reductases are repressed in the presence of oxygen. The sensitivity to oxygen differs per reductase, with nitrate reductase as the least sensitive one (Knowles, 1981).

Most denitrifying bacteria in soil are facultative anaerobes, which start to denitrify when oxygen is not available (Paul and Clark, 1996). Denitrifiers are mainly heterotrophic, so the decomposibility of organic substrates is an important control of denitrification (Knowles, 1981). Following from the above description, principle factors controlling denitrification are: the presence of denitrifiers and denitrification enzymes, nitrate levels, available organic carbon and the presence of anoxic conditions. Next to these, important factors are temperature and pH (Knowles, 1981). Factors regulating denitrification in soil are controlled in different and complex ways; the spatial and temporal variability of each factor is adding to the high variability of denitrification activity that is often observed in the field. In the following sections, the mentioned factors will be described in soil, with special emphasis on the development of anoxic conditions, as this is the main subject of this thesis.

**Decomposition stages in a humus form**

The litter layer in acid forest ecosystems is an accumulation of organic matter formed from input by litterfall. The accumulated organic matter layer represents a decay continuum, in which the age of organic matter increases with depth. Successive stages of decay are reflected by the presence of distinct horizons. Dickinson and Plugh (1974) described the following sequence for more humus forms in coniferous forests.
L freshly fallen, undecomposed needles
F1 dark brown intact, recognizable needles extensively colonized by fungi
F2 greyish, fragmented, compressed but recognizable needles containing hyphal fragments and animal faeces. Mesophyll collapsed
H humus-like amorphous mass of animal faeces and needle and microbial fragments
A an intimate mixture of humus and mineral soil

These horizons differ in chemical and physical properties of the organic matter, as well as in local microclimate (Dickinson and Plugh, 1974; Green et al., 1993). Such differences are induced by the activity of decomposing organisms. By changing properties of organic matter, one group of organisms creates new conditions for another group of organisms. In this way decomposition stages correspond with a succession of different communities of fungi, bacteria and microfauna. This succession is reflected by a stratification of these communities in different horizons (Zvyagintsev, 1994; Berg, 1997). Ponge (1991a) studied the succession of fungi and fauna in pine litter. He observed several stages of fungal decomposition with fungi colonizing needle surfaces and penetrating needle interiors in increasing degrees. Hereafter, he noticed a stage of faunal decomposition in which needle interiors were penetrated by micro- and mesofauna or entire pieces of needles ingested and compacted to faeces by macrofauna. Moreover, Ponge (1991a) found that bacterial development is associated with activity of fauna, as he only observed bacteria in needle interiors when needles showed signs of faunal attack. Furthermore, bacteria generally proliferated in faecal pellets inside and outside needles. In a more advanced stage, when pine roots developed in the layer of old needles, mycorrhizal fungi seemed to impede further bacterial development.

1.2 Anoxic conditions in soil

Low to zero oxygen concentrations have not been demonstrated directly in forest floors on well drained soils. However, the observation of strictly anaerobic microbial processes, such as methanogenesis, suggests that regions with zero oxygen concentrations must occur in aerobic forest floors (Musacchio et al., 1996; Yavitt et al., 1995). In contrast to forest floors, the occurrence of anoxic conditions has been studied extensively in mineral soils and other environments, such as biofilms and marine sediments (Sexstone et al., 1985; Binnerup et al., 1992; Dalsgaard and Revsbech, 1992; Sierra and Renault, 1996). In the following sections I will summarize reported research on anoxic conditions in mineral soils regarding theory, experimental results and modelling exercises. Knowledge of oxygen dynamics in mineral soils forms a basis for the present study, as it enables interpretation of our results obtained in a forest floor. Furthermore, differences between oxygen dynamics in forest floors and mineral soils are relevant for the understanding of the entire forest soil system.
Development of anoxic conditions
The oxygen concentration in soil results from consumption of oxygen on the one hand and a supply of oxygen from the atmosphere on the other hand. Oxygen supply in soil has been demonstrated to occur mainly by gaseous diffusion (Currie, 1961). To maintain high oxygen concentrations in oxygen consuming soil, the oxygen diffusion path should mainly exist of continuous air-filled pores (Currie, 1965). In unstructured soils, there may be one continuous pore phase; in such soils, gradients in oxygen concentration will be one-dimensional and related to profile depth. In structured soils, a more complex diffusion pattern exists, as the development of soil structure may lead to the formation of different pore phases. For instance: there may be one phase of large pores between soil-structural units (interaggregate pores) and one phase of small pores within structural units (intra-aggregate pores) (Currie, 1965). Different pore phases often do not contribute equally to gaseous diffusion in soil (Currie, 1965). In well-drained soils, water is mostly held in the intra-aggregate pores and gaseous diffusion mainly occurs through interaggregate pores. Oxygen consuming microbial organisms often live in small pores, which are part of the intra-aggregate porosity (Paul and Clark, 1996). Consequently, to understand oxygen concentration dynamics following from oxygen consumption, oxygen diffusion should be studied within the soil aggregates as well as in bulk soil (Currie, 1965).

Measurement of anoxic conditions in soils
Anoxic conditions in aggregates
The presence of low oxygen concentrations in soil aggregates of different sizes depends on the diffusivity of oxygen in these aggregates and on oxygen consumption rates. As soil aggregates often are cemented to a certain degree, and moreover have a higher water content and a lower porosity than the bulk soil, the diffusivity of these aggregates will be lower than that of the bulk soil (Currie, 1961). Measured diffusivities in soil aggregates range from $2 \times 10^{-8}$ to $1 \times 10^{-12}$ m$^2$ s$^{-1}$ (Greenwood and Goodman, 1967; Sextone et al., 1985; Zausig and Horn, 1990; Højberg et al., 1994; Sierra et al., 1995; Sierra and Renault, 1996; Rappoldt, 1995). The effective diffusivity generally decreases with increasing water content of aggregates and decreasing porosity (Zausig and Horn, 1990). Regarding the effect of water content, not only the amount of water itself is of importance, but also the distribution within the aggregate. Leffelaar (1986) observed that upon wetting of soil aggregates, water was mainly distributed in the outer shell of an aggregate. As a consequence, diffusion occurred only in the water phase, leading to a lower diffusivity of oxygen than was expected from the water content of the whole aggregate. Another factor influencing diffusivity is aggregate structure; root channels or subaggregate surfaces can function as pathways for diffusion (Sierra and Renault, 1996).

Sierra and Renault (1995, 1996) observed oxygen consumption rates in aggregates that decreased with aggregate size. The main reason for this was that oxygen consumption appeared to be positively affected by oxygen concentration, indicating
first order oxygen consumption kinetics with respect to oxygen concentration. Since oxygen concentrations in larger aggregates were lower than in smaller ones, due to the larger diffusion path, oxygen consumption rates were also lower than in smaller aggregates. However, first order oxygen consumption may not be a general phenomenon in aggregates, as Greenwood (1961) observed no dependence of oxygen consumption on oxygen concentration in his experiments. Apart from a possible dependence on the actual oxygen concentration, oxygen consumption also depends on carbon availability and microbial activity in aggregates. For instance, Zausig and Horn (1992) measured steeper oxygen concentration gradients in aggregates from the humic A-horizon of a Vertisol than in aggregates from the B-horizon, which was poor in organic matter. The microbial activity within aggregates was related to the organic matter content and has been reported to be inhomogeneously distributed or to decrease towards the center of aggregates (Zausig and Horn, 1992; Sierra and Renault, 1995, 1996).

The oxygen diffusivity and consumption of the refered mineral aggregates led to anoxic conditions in aggregates at radii ranging from 4 to 20 mm (Sexstone et al., 1985; Højberg et al., 1994; Sierra and Renault, 1996). Radii of these aggregates are larger than those of organic particles of needles and leaves in a forest floor. Whether anoxic conditions can develop in organic particles, despite their small diameter, depends on the diffusion coefficient and microbial activity. These have not been measured so far. Nevertheless, in a decomposing clover leaf of only 0.3-0.4 mm diameter anoxic conditions were demonstrated (Højberg et al., 1994), which suggest that anoxic conditions are possible in small organic particles. However, anoxic conditions in the clover leaf were attributed to high oxygen consumption rates related to the high availability of easily decomposable organic matter. Similar oxygen consumption rates may not be expected in litter particles of several years old. Since actual oxygen consumption rates and diffusion coefficients in litter are unknown, experiments must make out whether anoxic conditions are possible. For this aim, the use of oxygen microelectrodes seems an appropriate measure.

Anoxic conditions in soil profiles
The complexity of oxygen concentrations in soil profiles very much depends on soil structure. In unaggregated soils, the oxygen concentration gradients may be mainly vertical, with the air-filled pore content, oxygen consumption rate and the distance from the surface as the main factors determining oxygen concentration at a certain depth. In aggregated soils, a similar vertical gradient may occur, in combination with radial concentration gradients within aggregates. However, structured soils do not always behave like a homogeneous distribution of aggregates within a macropore phase. Indeed, inhomogeneous distribution of soil water led to local water saturation and discontinuity in interaggregate pores in soils (Greenwood and Goodman, 1967). This resulted in oxygen concentrations lower than expected from bulk water content. Furthermore, inhomogeneously distributed high microbial activity led to anoxic microsites that were not related to the distance from macropores in a clay soil (Rappoldt, 1992).
Not much is known about oxygen concentration gradients in litter layer profiles. Dickinson and Plugh (1974) cited a study in which oxygen concentration was at an atmospheric level throughout the profile. However, at high water contents oxygen diffusivity decreases in forest floors, and may lead to vertical oxygen concentration gradients (Freijer, 1994a). When concentration gradients do develop in the litter layer profile they are probably related to the sequence of humus form horizons. These horizons show distinct differences in structure due to decomposition by soil flora and fauna.

**Modelling anoxic conditions in soil**

Numerical models of oxygen diffusion in soils are important tools as they can be used to estimate the soil anoxic volume. Quantifying the anoxic volume is relevant since anoxic conditions give rise to anaerobic microbial processes and negatively affect plant growth. The importance of oxygen diffusion in the aeration of soils, and therefore plant growth, has long been acknowledged in agricultural science. Various early studies related oxygen diffusion to soil porosity and or air-filled porosity (Penman, 1940; Millington, 1959). Since then, such relations between soil diffusivity and porosity have been improved and extended with empirical parameters related to soil type or soil pore properties (Freijer, 1994a; Jin and Jury, 1996; Moldrup et al., 1996). However, models of oxygen diffusion in interaggregate pores do not account for the quantification of oxygen in soil aggregates. Therefore, models have been developed to predict oxygen diffusion and uptake within and between soil aggregates (Leffelaar, 1979; Smith, 1980). In these models soil structure is represented by a hypothetical aggregate size distribution. Furthermore, a clear distinction between intra-aggregate pores and interaggregate pores is applied. Leffelaar (1979) also implemented the distribution of water in interaggregate pores. The effect of water in interaggregate pores is that part of the aggregate surface area is blocked for oxygen diffusion from air-filled pores. Renault and Sierra (1994) demonstrated that the assumptions on which distribution of water was implemented greatly affected calculated anoxic volumes. Furthermore, they showed in their calculations that the effect of water content highly depended on soil structure, soil temperature and microbial activity.

The way soil structure is represented in a diffusion model appeared to be of great influence on simulated anaerobic volumes (Arah and Vinten, 1995). Therefore, soil structure needs to be quantified for the prediction of realistic anoxic volumes with aggregated models. Rappoldt and Verhagen (1999) developed a method to quantify

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1 The term litter layer differs from humus form as the litter layer comprises only the organic horizons of a forest soil while the humus form refers to these organic horizons as well as the Ah horizon (mineral topsoil with high organic matter content) (Green et al., 1993)
soil structure from thin sections. In thin sections distance distributions were measured from points in soil aggregates to the nearest interaggregate pore.

The above simple or complex models for aggregated soils are still a simplification of soil structure and cannot be used if the soil under consideration does not behave like an aggregated soil (Rappoldt and Verhagen, 1999). In such soils there is no clear distinction between intra- and interaggregate pores, which can be the case for a clay soil (Rappoldt, 1992). Modelling soil anoxic volume without the concept of aggregated soils can be done with fractal models, which have the advantage that fractal dimensions of soil can be measured (Peyton et al., 1994; Anderson et al., 1996). Rappoldt and Verhagen (1999) showed that in a fractal model soil, the number of pores unconnected to the hypothetical soil surface greatly affected the simulated anoxic soil volume. When modelling exercises are performed in studies on oxygen diffusion-reaction in the forest floor, it is relevant to determine whether forest floors can be considered as aggregated systems. If they can, this allows the use of models for aggregated systems presented in the literature.

1.3 Measured relation between denitrification and anoxic conditions in soils

Relations at the aggregate scale

At the aggregate scale the relation between denitrification and local oxygen concentration level has been studied in a direct way by the use of oxygen and/or nitrous oxide microelectrodes (Sexstone et al., 1985; Højberg et al., 1994). Sexstone et al. (1985) found that denitrification in aggregates was always associated with anoxic sites, but not all aggregates with anoxic sites showed denitrification activity. This observation may typify denitrification in aggregated soils in general. Parry et al. (1999) found a larger denitrification activity in soils with large aggregates in arable soil than in the smaller aggregates under pasture, which they explained by larger anoxic microsites within the larger aggregates. In contrast, Seech and Beachamp (1988) actually found an inverse relation with denitrification activity and aggregate size, which was caused by a low organic carbon availability in larger soil aggregates. Moreover, they concluded that denitrifying bacteria were mainly present in the outer, oxic parts of aggregates. Low denitrification activities in anoxic sites within aggregates were also found by Højberg et al. (1994). Indeed, calculating diffusion rates of organic carbon and nitrate within mineral aggregates, Myrold and Tiedje (1985b) concluded that denitrification in mineral soil aggregates was limited by substrate in aggregates larger than a critical size of 2 mm. The above presented studies imply that anoxic conditions are not always the most important limiting factor for denitrification at the aggregate scale.

Relations at the field scale

Field denitrification rates are usually measured as surface or soil column fluxes of nitrous oxide using the acetylene inhibition technique. Direct relations between
denitrification and anoxic (forest) soil volumes could not be demonstrated since it has not been possible to precisely quantify anoxic volumes in the soil compartment. Nevertheless, the importance of anoxic conditions in the field is confirmed by correlations between denitrification rates and environmental factors mediating anaerobiosis in the soil. First, increased denitrification rates have been measured at high water contents or water filled pore space (Robertson and Tiedje, 1984; Davidson and Swank, 1986; Carnol and Ineson, 1999; Shelton et al., 2000). Water content depends on soil texture, structure, drainage, groundwater level, precipitation, irrigation, thaw of frozen soil water or snow. Second, denitrification rates have been found to correlate with factors affecting oxygen consumption rate, such as carbon availability, microbial biomass and temperature (Robertson and Tiedje, 1984, Groffman and Tiedje, 1989a,b; Drury et al., 1991; Mogge et al, 1998).

Relations between field denitrification rates and environmental factors are most clear when yearly fluxes are used and relevant site factors, which are stable in time. For instance, Groffman and Tiedje (1989a,b) found that nearly 90% of the yearly denitrification of different sites could be explained by the factors drainage and texture. By contrast, Robertson and Klemmedsson (1996) found that momentary denitrification rates did not show consistent relationships with environmental factors.

Studies on field denitrification and relations with environmental factors in forest soils seldom specifically address the forest floor. Those that do, show that enhanced denitrification rates are often associated with high water contents (Musacchio et al., 1996; Papen and Butterbach-Bahl, 1999), which may induce anoxic conditions. Laboratory studies with litter confirm that anoxic conditions are an important stimulating factor as denitrification was one or two orders higher with anaerobic incubations than with aerobic incubation (Pang and Cho, 1984; Laverman, 2000). Since forest floors significantly contribute to cumulative surface fluxes of denitrification products (Martikainen et al., 1993; Musacchio et al., 1996; Dong et al., 1998; Regina et al., 1998; Papen and Butterbach-Bahl, 1999), extensive research is needed for further insight in the dynamics of denitrification in forest soils.

1.4 Other factors regulating denitrification

Presence and activity of denitrifiers

Most denitrifiers in soils are facultative anaerobes and their activity in soil is mostly expressed under aerobic conditions by their ability to compete for carbon substrate with other heterotrophs (Myrold and Tiedje, 1985a). The amount of (facultative) anaerobes in soils is much less than that of aerobes (ca. 1-10 vs 90-99%, Küsel et al., 1999). The number of denitrifying bacteria in soils correlates with total numbers of anaerobes, moisture content and annual mean rainfall in a study with samples from different climatic zones and mineral substrates (Gamble et al, 1977). In individual forest soil profiles microbial biomass and potential denitrification rates decrease with depth along with organic matter decomposability or total organic matter.
content (Pang and Cho, 1984; Heinrich and Haselwandter, 1991). Furthermore, microbial activity and likewise, potential denitrification activity, have been found to be higher in the rhizosphere (Scott Smith and Tiedje, 1979) and microsites with a local high concentration of easily decomposable organic carbon (Parkin, 1987; Christensen et al., 1990a,b; Murray et al., 1995).

Due to their ability to respire under aerobic conditions, denitrifiers are present in almost any soil. As a consequence denitrification will hardly be limited by the presence of denitrifiers at the field scale (Lens et al., 1991). However, at the aggregate scale in a forest floor, denitrification was found to be limited by the activity of denitrifying bacteria, as denitrifying activity was absent in 7 out of 30 particles (faecal pellets). In addition, Ponge (1991a) observed that bacteria in the L and F layers of a litter layer were mainly present in individual communities restricted to microsites.

Although the number of denitrifiers in general shows a clear relation with total microbial biomass, several studies indicate that denitrifying bacteria occur in preference to other soil bacteria in soils that exhibit frequent periods of anaerobiosis (Groffman and Tiedje, 1989a,b). Struwe and Kjøller (1994) found the number denitrifiers in a waterlogged forest soil to be $10^3$ to $10^4$ times higher than in a well-drained forest soil. Furthermore, Philippot et al. (1996) demonstrated that bacteria capable of nitrate reduction were in advantage in colonising anoxic soil aggregate centers.

**Denitrification Enzyme Activity (DEA) in soils**

The reaction sequence of denitrification is catalyzed by the following reductase enzymes: nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase. These enzymes are repressed in the presence of oxygen. Activity of existing denitrification enzymes and de novo synthesis were shown to be negatively affected by a low pH (Ellis et al., 1998). Firestone et al. (1980) observed that an effect of pH on repression of enzymes only occurred in the presence of high nitrate concentrations. These enzymes have been shown to be persistent in soils, even after periods of desiccation (Scott Smith and Parsons, 1985). At the onset of anaerobiosis, synthesis of nitrate reductase is derepressed most quickly (within several hours); synthesis of nitrous oxide reductase is derepressed last (after 16-33 hours) (Firestone et al., 1980; Dendooven and Anderson, 1994). The different periods needed for derepression of synthesis of enzymes can lead to an accumulation of denitrification products which changes with the duration of anaerobiosis (Firestone et al., 1980; Weier et al., 1993; Dendooven and Anderson, 1994).

The concentration of existing nitrate reductase in soil is usually the highest and most persistent (Dendooven and Anderson, 1994). The concentrations of nitrate reductase and nitrous oxide reductase depend on the frequency of anaerobiosis in soil and on the antecedent water regime (Dendooven et al., 1996). Differences in initial
concentration of existing enzymes affect the initial accumulation patterns of nitrite, nitrous oxide and nitrogen gas in soils. The earlier mentioned derepression times subsequently determine the further development in accumulation pattern. Initial activity of existing denitrification enzymes is generally not much lower than the activity following *de novo* synthesis, several hours after onset of anaerobiosis (Scott Smith and Parsons, 1985). Activation and inactivation of enzymes by oxygen was therefore thought to be of greater significance for soils with transient periods of anaerobiosis than synthesis of new enzymes or growth of denitrifiers (Scott Smith and Parsons, 1985; Dendooven et al., 1996)

**Carbon availability**

The influence of organic carbon availability on denitrification in soils is two-fold. On the one hand carbon availability regulates denitrification directly by its effect on the activity of heterotrophic microbial biomass (Myrold and Tiedje, 1985a). On the other hand carbon availability affects denitrification indirectly when anoxic zones develop due to oxygen consumption during aerobic carbon decomposition. These two effects are strongly interrelated and are difficult to distinguish in experiments under aerobic conditions.

The effect of carbon availability solely on the activity of microbial biomass can be studied under anaerobic conditions (Swerts et al., 1996). Results of such studies indicate that denitrification increases with amendment of carbon in the order glucose > cellulose > leaves/needles> lignine (Rashid and Schaeffer, 1988). Swerts et al. (1996) studied the importance of indigenous carbon quality. They observed a decrease in denitrification rate in samples of the same soil containing carbon in an increasing stage of decomposition due to increasing periods of preincubation (3-21 days). The increase of denitrification rates upon carbon amendments under anaerobic conditions generally ranges from a factor 2-10 (Drury et al., 1991; Heinrich and Haselwandter, 1991; Swerts et al., 1996). The effect of carbon amendments increases if the incubation period allows for substantial microbial growth. This may explain increases of denitrification rates by a factor of at least 60 after amendment with glucose during incubation periods of eight days (Rashid and Schaeffer, 1987).

A disadvantage of studying carbon availability in anaerobically incubated samples is that denitrification will occur throughout the sample. In such samples denitrification occurs in zones that under field conditions may not experience anaerobiosis. If the activity or functionality of microbial populations differs in dependence of soil structure, anaerobic denitrification rates may be difficult to relate to denitrification rates that can be expected under field conditions.

Under aerobic conditions, the increase in denitrification rate upon carbon amendments can be much higher than the factor 2-10. The much stronger effect of carbon under aerobic conditions must be attributed to the development of anoxic conditions in zones with high carbon availability (Rice et al., 1988; Christensen et al.,
1990a,b; Breland, 1994; Jordan et al., 1998). Microsites with a high carbon availability and oxygen consumption rates allowing for the development of anoxic conditions have been referred to as “hot-spots” (Christensen and Tiedje, 1988; Parkin, 1987). An excellent way to study the effect of carbon availability is to measure simultaneously respiration rates, denitrification rates and dynamics in anoxic volume. This has been done in studies with manure applications in mineral soil and biofilms (Dalsgaard and Revsbech, 1992; Petersen et al., 1996).

**Availability of nitrate**

Nitrate concentrations have increased in forest soils with a high atmospheric N-deposition or with long-term applications of fertilizers (Van Breemen and Verstraten, 1991). In forest soils with an excess of nitrogen considerable amounts of nitrate are produced by nitrification (Van Breemen and Verstraten, 1991; Tietema et al., 1992a,b). In such “nitrogen saturated” forest ecosystems, nitrifiers do not have to compete for ammonium with tree and plant roots.

Nitrification rates have been found to correlate with potential denitrification rates (Robertson and Tiedje, 1984). Consequently, denitrification is limited by nitrate availability in forest soils with low nitrate concentrations and a low nitrifier activity (Bowden et al., 1990; Paavolainen and Smolander, 1998). Likewise, limitation of denitrification by nitrate availability also occurs in soils, that exhibit favourable conditions for denitrification, such as water-logged soils (Schipper et al., 1993; Delaune et al., 1998). In such soils, the demand for oxidators exceeds the available amount of nitrate.

Nitrate concentration affects the denitrification process in two ways. First, high nitrate concentrations increase the reduction rate of nitrate to nitrite. Due to the higher affinity for nitrate than for nitrous oxide in the reaction sequence, an accumulation of $\text{N}_2\text{O}$ is often observed in soils with high nitrate concentrations (Nømmik et al., 1984). Due to accumulation of nitrous oxide the $\text{N}_2\text{O}/\text{N}_2$ ratio is high in soils with high nitrate concentrations (Firestone et al., 1980). Second, high nitrate concentrations in soil solution increase the diffusive flux to zones with denitrification activity. The effects of nitrate concentration on the reaction rate itself and on the diffusive flux are difficult to separate in soil. The importance of nitrate concentration on the diffusive flux is illustrated by much higher optimal nitrate concentrations found in soil solutions than in pure laboratory cultures. For instance, Km values (Michaelis-Menten constants) in pure cultures are lower than 0.015 mM while Km values in soil range from 0.13- 1.2 mM (Betlach and Tiedje, 1981). In the litter layer studied in this thesis nitrate concentrations are high (ca 1 mM and higher). Therefore, limitation of denitrification by nitrate availability seems highly improbable.
**Temperature**

In the presence of organic carbon, nitrate and anoxic conditions, denitrification rates are correlated with temperature (Knowles, 1981). Relations between temperature and denitrification in the field are often obscured by other factors being limiting at favourable temperatures. Although denitrification rates decrease with decreasing temperature, substantial amounts of denitrification have been observed with snowmelt or thaw after frost (Goodroad and Keeney, 1984a; Tietema et al., 1991; Papen and Butterbach-Bahl, 1999). Temperature not only affects the reaction rates themselves, but also influences denitrification indirectly by a lowering of the oxygen solubility in water and an increase in the oxygen consumption rate at increasing temperatures (Paul and Clark, 1996).

**Acidity**

Denitrification rate has been found to be negatively affected by low pH, both in respect to enzyme kinetics as in growth and activity of denitrifying bacteria (Müller et al., 1980; Ellis et al., 1998). Müller et al. (1980) found a correlation of potential denitrification with pH in the range of 3.6-7. In other soils however, adaption of denitrifiers to local low pH (4) was observed (Parkin et al., 1985; Martikainen and de Boer, 1993). pH influences the N₂O/N₂ ratio in the presence of nitrate: at low pH (4.9) and high nitrate concentrations the main product of denitrification is N₂O (Firestone et al., 1980). In this regard, the N₂O/N₂ ratio in the litter layer studied in this thesis is expected to be high due to high nitrate concentrations as well as a low pH (pH(H₂O) L: 4.4; F: 3.7, Koopmans, 1996).

1.5 **Sources of nitrous oxide other than denitrification**

The most important source of nitrous oxide, apart from denitrification, is nitrification. Nitrification is the oxidation of ammonium to nitrate, during which nitrous oxide can be produced as a by-product. The amount of nitrous oxide produced due to nitrification is positively correlated with moisture content and negatively with pH, oxygen concentration and ammonium concentration levels (Blackmer et al., 1980; Martikainen, 1985; Goreau et al., 1980).

The relative contribution of nitrification to total nitrous oxide production varies significantly, from negligible amounts to nearly 100% (Martikainen, 1985; Musacchio et al., 1996; Kester et al., 1997b; Stevens et al., 1997; Mogge et al., 1998; Papen and Butterbach-Bahl, 1999). Denitrification has been reported to be the main source of nitrous oxide at water contents higher than those at field capacity, or during the wet season in dry tropical forests (Mogge et al., 1998; Rudaz et al., 1991; Davidson et al., 1993).

A complicating factor in studying sources of nitrous oxide is when nitrification and denitrification are coupled (Speir et al., 1995). This occurs when reduction of nitrate
during denitrification is directly dependent on the nitrate produced during nitrification. When contribution of denitrification is estimated by specific inhibition of nitrification, denitrification rates may be severely underestimated as the nitrate availability will drop without the continuous replenishment due to nitrification. Although most nitrous oxide production in soil is estimated to be produced by nitrifiers and denitrifiers, it is known that nitrous oxide can be produced by many fungi and yeasts (Bleakley and Tiedje, 1982; Shoun et al., 1992). In addition, nitrous oxide can be produced during dissimilatory nitrate reduction to ammonium (DNRA) and nitrate assimilation (Scott Smith, 1982). The contribution of fungi and yeasts to nitrous oxide production is not known as no specific inhibitors have been found to determine their activity with (Bleakley and Tiedje, 1982). Apart from biological sources, nitrous oxide can also be produced by chemical reduction of nitrite to nitrous oxide, denoted as chemodenitrification (Paul and Clark, 1996). Contributions to nitrous oxide production by processes other than denitrification and nitrification are unknown in the forest soils in the Veluwe, and further discussion is beyond the scope of this thesis.

1.6 Research approach: a combination of laboratory experiments and modelling exercises

Research questions

In the above sections I reviewed studies on the occurrence of anoxic conditions in soil, the estimation of soil anoxic volumes using models and the relation between anoxic conditions and denitrification. Furthermore, the regulation of denitrification by other factors than anoxic conditions was discussed. The review shows that there is an urgent need for information on the specific occurrence of anoxic conditions in forest floors and on the relation between anoxic conditions and denitrification. Therefore, I formulated the following research questions for a Douglas fir forest soil in The Netherlands.

A. Where do anoxic conditions occur in a Douglas fir litter layer?

B. What are the conditions of oxygen diffusivity and oxygen consumption under which anoxic conditions occur in a Douglas fir litter layer?

C. How can the anoxic volume of a litter layer be modelled in a simple manner?

D. What is the relation between anoxic conditions and denitrification in a Douglas fir litter layer?
Approach and outline of this thesis

In soil science, as well as environmental science in general, it is hardly possible to experimentally study a single process factor in isolation, without disturbing the system and therefore the process itself. Measured variables in experiments therefore, often represent the combined response to several process factors. To enable unequivocal interpretation of experimental results, process models are often used. With proces models the behaviour of single factors can be studied, which makes them suitable for testing research hypotheses derived from experimental results. Therefore, the combined use of models and experiments is regarded as a valuable approach in environmental studies.

Experimental and modelling designs depend on the research questions that are formulated and on the spatial and temporal aspects of the process under study. Since anoxic conditions in the litter layer may occur on the scale of a particle or a
litter layer, experiments and models applied in this thesis will be concentrated on these scales.

To answer the formulated research questions I studied oxygen concentration levels in organic particles and in a litter layer in response to soil structure, diffusivity, oxygen consumption dynamics and water content. In addition, I studied the response of nitrous oxide to prevailing oxygen concentration levels. Figure 1.1 gives a schematic representation of the experiments and models adopted. The letters A-D correspond to research questions that are specifically addressed.

*ad question A*
Direct measurements of oxygen concentration profiles in single organic particles as well as whole layers are performed with oxygen microelectrodes. This is done at various oxygen consumption rates and water contents occurring under field conditions. To link concentration measurements with soil structure, thin sections are produced from the samples used in the microelectrode experiments. (subject of chapter 2).

*ad question B*
Diffusivity and oxygen consumption dynamics in organic matter are estimated with additional microelectrode experiments. This is done by measuring oxygen concentration dynamics in an organic particle during a stepwise increase of the oxygen concentration outside the particle. Measurements are compared with results of diffusion-reaction models at different scenarios of diffusivity and oxygen consumption. Diffusion-reaction models are applied to the organic matter fabric of the litter layer thin section. (subject of chapter 3).

*ad question C*
With the results obtained in the preceding chapters, an attempt is made to model the anoxic volume of the organic layer in a simple manner, suitable for application in large-scale models. A method of simplifying structure of aggregated soils (Rappoldt and Verhagen, 1999) is tested for the forest floor. This is done by applying diffusion-reaction models on a generalized representation of a litter layer structure and on the unsimplified litter layer structure. (subject of chapter 4).

*ad question D*
To address this question, nitrous oxide production is measured in response to water content, oxygen consumption rate and nitrate availability. The relation with anoxic conditions is studied by simulating anoxic conditions in water-filled interparticle pores at the conditions of the experiment, and comparing the results with observations. In addition, limitation of denitrification in particles by nitrate diffusion is investigated with a diffusion-reaction model. (subject of chapter 5).

Finally, in a synthesis the results are extrapolated to other compartments of the litter layer, and as far as possible, to forest soils in general.
The research location is a Douglas fir stand in the Speulderbos, the Veluwe, in the central Netherlands (Figure 1.2). The study site is situated in a region with high atmospheric nitrogen deposition. The forest was planted in 1962 and has a tree density of 780 trees ha\(^{-1}\) without undergrowth (Figure 1.3). The soil is classified as an Haplic Podzol (FAO, 1988) and is formed on heterogeneous ice-pushed sandy loam deposits and loamy sand glacio-fluvial deposits. The humusform is classified as a Moder (Green et al., 1993). The soil is well-drained with a groundwater level at 40 m depth throughout the year. Average precipitation is 834 mm \(y^{-1}\) and is evenly distributed over the year.

The site has been used for on-going research in various disciplines during the last decade. Relevant studies in respect to this thesis were performed on: forest soil nitrogen mineralization and nitrification (Tietema, 1992; Koopmans, 1996), soil hydrology (Schaap, 1996), oxygen diffusion in soil (Freijer, 1994b), soil acidification and nutrient dynamics (Wessel, 1997).
The forest soil profile at the research location consists of a L, F and Ah horizon with a sharp transition between the organic layer and the mineral soil. An H horizon is absent. Below several microscopical observations are presented of Douglas fir litter from the study site, that illustrate the succession of fungi and microfauna in litter that was sketched in the general introduction (Figures 1.4 to 1.7). No images of bacterial development have been made, although the presence of bacterial microcolonies was observed on the surface of needles from the F2 horizon, after staining with dyes (DAPI (4',6'-diamidino-2-phenylindole-2-HCl) and FITC (fluorescein isothiocyanate)).
Table 1.1: pH\textsubscript{KCl}, net mineralization rate and nitrification rate measured in incubation experiments, copied from Tietema et al., 1992a. Values are averages and standard deviations (between parenthesis) based on 4 replicates (except L horizon). Only the first 5 cm of the mineral Ah horizon are analysed.

<table>
<thead>
<tr>
<th>pH</th>
<th>Net mineralisation rate</th>
<th>Nitrification rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>H\textsubscript{2}O\textsuperscript{a}</td>
<td>(mg N kg\textsuperscript{-1} 4 wk\textsuperscript{-1})</td>
<td>(g N m\textsuperscript{-2} 4 wk\textsuperscript{-1})</td>
</tr>
<tr>
<td>L</td>
<td>4.4</td>
<td>1380.3</td>
</tr>
<tr>
<td>F</td>
<td>3.7</td>
<td>299.9 (151.4)</td>
</tr>
<tr>
<td>Ah</td>
<td>3.8\textsuperscript{a}</td>
<td>19.1 (5.7)</td>
</tr>
<tr>
<td>total</td>
<td>1.91</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Koopmans (1996); pH in Ah horizon measured in first 10 cm.

The organic layer mass varies from 3.2 to 4.5 kg\textsubscript{dry} m\textsuperscript{-2} (Wessel, 1997). The organic soil is substantially acidified as is evidenced by pH values of 4.4 in the L horizon to 3.8 in the Ah (Table 1.1). Due to long-term atmospheric nitrogen deposition the soil has become nitrogen saturated, which means that the forest has reached its maximum capacity to retain nitrogen in soil and vegetation. Nitrogen saturated soils are characterised by high nitrogen mineralisation and nitrification potentials (Tietema et al., 1992a). Net mineralization rates and nitrification rates of the forest soil in Speuld are given in Table 1.1. Rates are measured in incubation experiments, in situ rates are about a factor 5 lower (Koopmans et al., 1995).
Figure 1.4: Fungal colonization of an intact needle (L). Thin section photograph by scanning electron microscopy. Bar = 8.77 μm.

Figure 1.5: Microfaunal penetration in needle interior (F2). Photograph by scanning electron microscopy. Bar = 43.7 μm.
Figure 1.6: Deposition of microfaunal faeces inside needle (F2). Thin section photograph by light microscopy. Scale: 1 cm = ca. 0.15 mm.

Figure 1.7: Activity of fungi inside needle (F2). Thin section photograph by light microscopy. Left: overview of needle, scale: 1 cm = ca. 0.1 mm; right: close-up of fungi, scale: 1 cm = ca. 0.02 mm.