Predicting soil Nitrogen supply

relevance of extractable
soil organic matter fractions

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Abstract
Predicting the potential of soils to supply N is of considerable importance to maximize agricultural N use efficiency and to minimize environmental losses. This research examines and evaluates the current soil testing approach, which uses extractable organic N (EON) fractions to predict soil N supply, using isotopic $^{15}$N tracing, multivariate statistics and meta-analytical techniques.

Almost all 20 EON fractions that have been developed during recent decades significantly reflect the potential of soils to supply N, in spite of the strong differences in size and composition of EON due to extraction methodology. The EON fractions have therefore been considered as highly bio-available N pools in soil. However, most of them performed either worse than or similarly to total N as predictor of soil N supply, and the uncertainty of the predicted soil N supply (even under controlled environmental conditions) is still too big for serious improvement of fertilizer management.

A micro-diffusion method is developed to estimate gross EON fluxes in order to investigate the biochemical basis for observed relationships between EON and soil N supply. The fate of EON fractions in N mineralization, in particular those fractions that are obtained with weak hydrolyzing salt solutions, is comparable to that of dissolved organic N (DON). Both DON and EON can be considered as (intermediate) decomposition waste products in an abiotic and biotic controlled equilibrium with total N. Therefore, their relationship with soil N supply likely reflect that both DON, EON, and soil N supply are mutually dependent on total N.

The dependency of soil N supply on methodological and environmental issues strongly encourages more effort to be put into validation and up-scaling, particularly regarding the quantification of the differences between laboratory and field experiments. A combination of soil testing with simulation modeling is necessary to account for the numerous environmental factors controlling soil N supply. The exact EON fraction that can be used in such an approach is less important and practical considerations may be decisive to select one for routine application in soil analysis.

In conclusion, a holistic approach, which considers spatial and temporal variability of both soil N supply and crop N demand, may provide a successful approach to improving fertilizer management at the farm-scale.
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CHAPTER 1

1

GENERAL INTRODUCTION

Gerard H. Ros
Chapter 1  
Predicting soil N mineralization

1.1 Relevance of this thesis

Nitrogen (N) is often the growth-limiting nutrient in agricultural ecosystems. The N taken up by crops annually can range between 100 and 300 kg N ha\(^{-1}\) (Pronk & Groenwold, 2004; Velthof et al., 2009) and is derived from a number of sources, particularly from synthetic fertilizers, biological N fixation, and mineralization of soil organic matter (SOM), crop residues and manures (Keeney, 1982; Curtin & Campbell, 2007). The contribution of mineralization to crop N supply may range from less than 20 to more than 200 kg N ha\(^{-1}\) depending on the quantity and quality of mineralizable organic N in the soil and the environmental conditions that control the rate of mineralization (Cadisch & Giller, 1997; Brady & Weil, 2002). Estimating the N supply of a soil is therefore of considerable importance to maximize agricultural N use efficiency and to minimize environmental losses.

Nitrogen losses from soils to the environment occur in the form of NH\(_3\) due to volatilization, in the form of N\(_2\)O, NO or N\(_2\) produced during denitrification and in the form of NO\(_3\) due to leaching (Oenema et al., 2007). The contribution of these N losses to eutrophication, ground water quality and global warming are of great concern to our environment (e.g., Hansen et al., 2001; Galloway et al., 2003). In response to the environmental impact of N emissions, a series of governmental policies and measures have been implemented at both national and international levels. Recent surveys show that these measures have already had a positive effect on NO\(_3\) levels in numerous water courses (European Commission, 2007) and led to a reduction in the use of commercial inorganic N fertilizer in most European countries (Faostat, 2010). Nevertheless, agriculture still accounts for significant N emissions; yearly losses in the Netherlands have been estimated to be 288 kg N ha\(^{-1}\) agricultural land (Velthof et al., 2009), accounting for approximately 62% of the annual inputs.

Balanced and sustainable farm-scale N management (matching N supply with N demand) can significantly reduce N leaching losses to the environment by about 30% (Olfs et al., 2005; Wivstad et al., 2005; Velthof et al., 2009). Opti-
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Minimisation of N management also has large synergetic effects, because NO$_3^-$ leaching, NH$_3$ emission, and N$_2$O emission all decrease when the input of N decreases (Velthof et al., 2009). The potential effect of sustainable fertilizer management on N losses is therefore large, in particular for most European countries, where the mean amount of available N (originating from both fertilizer N and soil N supply) exceeds the crop needs. However, sustainable fertilizer management requires a thorough assessment of the N supply originating from the soil and from organic amendments.

The potential of soils to supply inorganic N can be assessed by biological incubation methods, chemical extraction methods, and simulation modelling (Griffin, 2008; Nannipieri & Eldor, 2009; Manzoni & Porporato, 2009). In spite of the significant efforts made by these methods and modelling tools during last decades, one of the major goals of soil N research, that of being able to predict soil N supply and fertilizer N needs, has not been achieved (Nannipieri & Eldor, 2009). The current economic and environmental concerns therefore continue to reinforce the need for routine methods estimating soil N supply.

1.2 Estimating soil N supply

**Biological methods** essentially measure a mineralizable fraction of soil organic N responsible for the production of inorganic N through microbial activity. Biological methods measure this mineralizable N fraction directly by determining net N mineralization or crop N uptake in lab, greenhouse or field experiments. **Chemical methods** are designed to isolate chemically labile from recalcitrant organic matter. The chemically labile fraction may represent the most biologically available fraction of SOM. These methods extract a specific organic N or organic carbon (C) fraction based on its solubility in water with and without electrolytes, its hydrolysability with water or acids, and its resistance to oxidation. **Simulation models** quantitatively describe the processes controlling N supply in soil using fundamental or empirical equations (Jansen, 1984; Smith et al., 1997, Manzoni & Porporato, 2009). Each of the three approaches has its own advantages and disadvantages.
Biological methods currently reflect the microbial processes in the soil better than the two other methods, but they are time-consuming, labour intensive and their outcome strongly depends on experimental conditions (Keeney, 1982; Cabrera et al., 2005). Chemical methods estimate ‘what can be mineralized’ based on the characteristics of the organic matter present, and they are therefore not able to integrate the numerous interrelated soil, plant, environment and management factors which control the actual N release and plant growth (Bremner, 1965). However, they may reflect a certain potential of a soil to release N, and soil analysis is often simple, rapid, and reproducible. Simulation models account for the complex processes in soil, including the interactions between organic matter, soil texture, microbiology and environmental conditions. However, their current performance is often not good enough for on-farm application. They also require site-specific calibration, which requires numerous input variables that are often unknown or difficult to obtain.

1.3 Chemical methods estimating soil N supply

1.3.1 Background and validity of a chemical approach

Efforts to develop chemical methods for the estimation of soil N supply have a long history (reviewed by Bremner, 1965; Keeney, 1982; Griffin, 2008), despite Walksman (1936) noting that ‘any attempt to divide soil organic matter on the basis of its practical utilization would prove to be largely artificial’. All chemical methods release a distinct SOM fraction from the soil, which amount and characteristics depend on the salt type, the molarity of the solution, the soil-to-solution ratio, and the duration and temperature of the extraction method used (Zsolnay, 2003). When referring to this extractable SOM fraction, I use the abbreviations EOC and EON for extractable organic C and N, respectively.

The number of papers evaluating the predictive value of these EOC and EON fractions in order to predict soil N supply has exponentially increased over the last 100 years and is still increasing. Unfortunately, the results of these papers are often contradictory (Keeney, 1982; Griffin, 2008), highlighting the need for a more fundamental understanding of the underlying mechanisms affecting
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both EON and the potential of soils to supply N. It may even raise the question whether chemically based extraction methods are able to selectively extract a bioavailable fraction of soil organic matter (Bundy & Meisinger, 1994).

There are several reasons why the debate on the validity of a chemical approach of soil N mineralization is still unresolved. First, the identification of an appropriate chemical method is hampered by huge variation in calibration conditions (e.g. duration, temperature, soil pre-treatment) and the lack of standard protocols for the assessment of EON fractions in soils. Because both the EON fraction and the soil N supply are affected by methodological issues, the strong variation among studies hampers meaningful comparison of their results. Second, little attention has been paid to the reliability of the biological reference method from which the predictive value of the chemical method has been inferred (Wang et al., 2001). Third, both the EON fraction and the soil N supply have shown temporal and spatial variability where climatic, soil and farming conditions strongly differ among studies (Moisier et al., 2004). Fourth, validation of calibrated chemical methods is rare, limiting their applicability to the dataset from which they are derived. Fifth, most studies evaluating the predictive value of soil tests to estimate soil N supply show few innovations: the majority of research deals with minor variations of pre-existing methods, using simple linear regression techniques, whereas the power of multivariate analysis and meta-analytical techniques has been overlooked. Similarly, only a few studies tried to integrate the chemical soil test approach with dynamic modelling tools (e.g., Campbell et al., 1997).

Most important however, is the substantial uncertainty that exists on the role of EOM fractions in the N cycle, while mechanistic approaches that explore the biochemical basis for observed relationships are rare (Kelley & Stevenson, 1985; Stockdale & Rees, 1994). This knowledge may help to identify the conditions under which the EON fractions can be used to improve fertilizer recommendations, or to design better methodologies.
1.3.2 The biochemical basis underlying the chemical approach

Three mechanisms have been postulated that may explain the often observed correlation between EON and the capacity of soils to supply N. The most common explanation postulates that EON fractions refer to a bioavailable N pool in soil, being formed through depolymerisation of soil organic matter and functioning as a labile source of inorganic N (Haynes, 2005). A higher value of EON indicates, in that case, an increase in bioavailable organic N and subsequently in the potential of soils to supply N. This bioavailable pool may be present in a field moist soil or may be created by soil pre-treatments such as drying and sieving in the lab, followed by extraction (Stockdale & Rees, 1994; Haynes, 2005). In a second explanation of the underlying mechanism of the chemical approach, it is assumed that EON fractions reflect the size of the microbial biomass (Juma & Paul, 1984; Kelley & Stevenson, 1985), which in turn depends on the size and quality of the organic matter. This explanation assumes that the size of the microbial biomass is indicative for the rate of net N mineralization (Jenkinson, 1968). Recent insights on mineralization pathways in soils indicate that organic compounds have to become dissolved before they are mineralized by microbes (Chapin et al., 2002; Schimel & Bennett, 2004; Geisseler et al., 2010). The production of this dissolved organic N (DON) pool is recognized as the rate-limiting step in N mineralization, and hence, the concentration of DON may be indicative for the net N mineralization rate (Haynes, 2005). This concept may give the third explanation of the underlying mechanisms of the chemical approach, but it is only valid for those extraction methods that collect an organic N fraction similar to that fraction present in soil solution (e.g., CaCl₂, KCl, and water).

Each of the three mechanisms have been criticized, but reliable techniques that quantify gross production and consumption rates of DON and EON are required to underpin the proposed mechanisms (McDowell, 2003). The various mechanisms are also not necessarily mutually exclusive when we take into account that the dissolved and extractable organic fractions consist of a heterogeneous mixture of compounds with distinct characteristics.
1.4 Fate of dissolved and extractable N in soil

1.4.1 Introduction and terminology

Understanding the fate of DON and EON in soil N mineralization may help to identify the underlying mechanism that explains the relationship between EON and soil N supply. It may also help to identify the chemical method with the highest predictive value (to predict soil N supply) and the conditions under which this method can be applied to improve fertilizer management (e.g., issues related to time scale of prediction, incorporation of fertilizer history, sampling time, etc). In my thesis, I distinguish between dissolved and extractable organic N because the methods to obtain these fractions are principally different (Zsolnay, 1996; 2003). With dissolved organic N, I indicate the organic N fraction that is present in the soil solution with a size less than 0.45 μm. With extractable organic N, I indicate that fraction of soil organic N that is released by soil extraction. From the more than 20 EON fractions that have been investigated, I primarily focus on the organic N fractions extractable with CaCl₂, K₂SO₄, and hot water.

1.4.2 Methodological aspects

Dissolved organic N is determined in collected soil water and operationally defined by using filtration with a specified filter size (0.45 μm; Zsolnay, 2003). Commonly used sampling devices collecting soil water are porous cups, porous plates, capillary wicks, resin boxes, and lysimeters (reviewed by Weihermüller et al., 2007). Some techniques determine DON in soil water collected from drainage pipes whereas others collect soil solution with artificial leaching or by centrifugation of field-moist samples (Giesler & Lundström, 1993; Raber, 1998). The concentration and the characteristics of the DON collected will depend on the sampling technique because both the concentration and the quality of DON depend on its location in the soil profile (Zsolnay, 1996). Extractable organic N is typically obtained from soils by shaking with water or salt solutions at a specified soil weight-to-solution volume ratio for short periods of time (1-3h), followed by separation of the solution phase by filtering and/or centrifugation for subse-
sequent analysis. A wide range of laboratory extraction procedures have been proposed for EON. These protocols differ in their use of extraction solvent, shaking time, temperature, soil preparation and method of analysis (Haynes, 2005). Both dissolved and extractable organic N are obtained indirectly by subtracting measured inorganic N from total dissolved (TDN) or extractable N (TEN).

1.4.3 Fate of DON and EON in soil N cycling

Dissolved organic N is increasingly recognized as important with respect to the soil N cycle. First, it is a major contributor to leaching of N in natural and agricultural ecosystems (Qualls, 2000; Van Kessel et al., 2009). Second, it plays a pivotal role in N mineralization and immobilization, probably representing the bottleneck in the biological soil N cycle (Schimel & Bennett, 2004; Jones et al., 2004). Lastly, it is an N form that can be taken up by plants directly, representing a possible ‘short circuit’ in the terrestrial N cycle (Neff et al., 2003). Extractable organic N likely has a different role in the soil N cycle, since it partly consists of organic matter that is not dissolved in situ at the time of sampling. When EON is obtained with weak hydrolyzing salt solutions or water, it may have the same fate and function as DON. When EON is obtained with stronger hydrolyzing salt solutions, it might be the major source of DON (Kalbitz et al., 2000). All EON fractions are proposed to reflect the soils’ potential to supply N (Keeney, 1982; Griffin, 2008).

1.4.4 Origin of DON and EON

Dissolved organic N in soil solution is found in different pore size classes, ranging from mobile water in cracks and channels to immobile water within aggregates. It can enter the soil as soluble organic material leached from fresh organic matter residues (e.g., manure, litter). Furthermore, it can be generated within the soil through decomposition of solid organic matter, and by the release of both microbial metabolites and plant root exudates (Qualls, 2000; Haynes, 2005). The contribution of the different sources is under debate (Kalbitz et al., 2000; Chen & Xu, 2008). Extractable organic N includes not only DON, but also other compounds that are released during extraction. These additionally released com-
pounds originate from lysis of microbial cells and desorption of organic compounds from solid surfaces (Zsolnay, 2003). The contribution of the additionally released microbial cell material or desorbed organic compounds may vary, dependent on the extraction methods used.

1.4.5 **Nature of DON and EON**

Dissolved and extractable organic matter fractions consist of a wide range of organic compounds, including simple aliphatic organic acids, phenols, phenolic acids, free amino acids, amino sugars, carbohydrates, and complex humic molecules of various molecular weights (Stevenson, 1994). In agricultural soils, free amino acids and amino sugars only account for <5% of DON, heterocyclic N bases up to 15%, and peptides and proteins for 35-57% (Murphy *et al*., 2000; Jones *et al*., 2004). The composition of EON fractions obtained with CaCl₂ and K₂SO₄ are approximately similar to that of DON: the main constituents are peptides and proteins (Matsumoto *et al*., 2004). Hot water EON is largely a mixture of carbohydrates and proteins (Balaria *et al*., 2009). The composition of both DON and the three EON fractions is strongly affected by methodology (Zsolnay, 2003; Jones *et al*., 2006; Haynes, 2005), but they have on average a relatively high molecular weight, and are relatively recalcitrant in nature. Characterization of specific compounds or functional groups within DON or EON can be helpful to elucidate their fate in soil, but it is not yet clear how the composition or characteristics of DON and EON relate to their functionality in soil N dynamics (Zsolnay, 2003).

1.4.5 **Factors that may control the dynamics of DON and EON**

Although the concentration of DON typically accounts for only 0.1 to 2% of total N in agricultural soils, it is considered by many authors to be the most dynamic and bio-available organic matter pool (Haynes, 2005). In contrast, concentrations of EON obtained with hot water can account for more than 10% of soil organic N. Both DON and EON are mainly controlled by similar processes (Kalbitz *et al*., 2000; Qualls, 2000), although their dynamics may differ. The main processes include mineralization and immobilization, adsorption and desorption,
and precipitation and solubilisation. A list of controlling factors that have been studied in recent decades is presented in Table 1.1.

Table 1.1. Factors that may control the dynamics of DON and EON, arranged over four different groups of parameters: molecular characteristics, soil solution properties, and external factors.

<table>
<thead>
<tr>
<th>Group</th>
<th>Controlling factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular characteristics</td>
<td>Elemental composition, size, structure, acidity, aromaticity, polarity, hydrophobicity</td>
</tr>
<tr>
<td>Soil solution properties</td>
<td>pH, concentration and type of cations and anions, O₂ concentration, flow rate, presence biofilms</td>
</tr>
<tr>
<td>Soil properties</td>
<td>Clay content, amount of oxyhydroxides, CEC, organic matter content, C-to-N ratio, pore size distribution, microbial density and composition</td>
</tr>
<tr>
<td>External factors</td>
<td>Temperature, moisture, water flow, season, drought &amp; rewetting, land use, crop species, soil management, fertilizer management, liming, soil fauna, deposition</td>
</tr>
</tbody>
</table>

In short, the dynamics of DON and EON in soils are affected by the molecular characteristics of the organic compounds involved, the solid phase properties, the microbial community and soil fauna, external factors such as the temperature and rainfall regime, and anthropogenic factors like soil and nutrient management (Kalbitz et al., 2000; Chantigny, 2003; Marschner & Kalbitz, 2003; Cookson et al., 2004; Chen & Xu, 2008). Despite intensive research in the last decade, our knowledge of the formation and fate of DON and EON in soils and their response to changing environmental conditions is still fragmented and often inconsistent (see, for example, the recent debate on the (a)biotic mechanisms responsible for the production of DON; Kemmitt et al., 2008; Kuzyakov et al., 2009).

1.5. Objectives of this thesis

Knowledge of the fate of DON and EON in soil N mineralization is of importance for development of fertilizer recommendation systems based on chemical extraction methods, ideally in combination with simulation models. This thesis therefore aims to explore the underlying mechanisms that are responsible for the ob-
served relationships between EON fractions and mineralizable N. Recalling the debate on the validity of a chemical estimation of mineralizable N, there is also a strong need to innovate the current soil test approach and to open new perspectives in this field. Knowing these challenges, I aim to evaluate the chemical soil test approach using isotopic $^{15}$N tracing techniques, multivariate statistics and meta-analytical techniques. More specifically, my objectives are:

- to evaluate all common chemical extraction methods for their ability to estimate the potential of soils to supply N;
- to quantify the influence of methodology, soil characteristics, environmental factors and nutrient management on the concentration of DON and EON in soils;
- to investigate whether and how dissolved and extractable organic N fractions are involved in N mineralization using $^{15}$N tracing;
- to evaluate the importance and applicability of the organic N fractions to improve N fertilizer strategies at the farm-scale.

1.6. Experimental approach and thesis outline

Two main topics are present in my thesis. The first main topic deals with research on the fate of DON and EON in soil, while the second deals with the relevance and potential of these fractions for accurate prediction of (potentially) mineralizable N (Fig. 1.1). The next chapter examines the influence of methodological and environmental factors on the concentration of DON and EON in soils using a meta-analysis approach (Chapter 2). After that, I review and evaluate results of chemical extraction methods that have been used to estimate (potentially) mineralizable N, again using the meta-analysis approach (Chapter 3). The variation in mineralizable N of ninety-eight Dutch agricultural soils and its relationship with EOM fractions and other soil properties is examined in Chapter 4 using multivariate statistical modelling. To further understand the role and functionality of organic N fractions in soil, I developed and tested a micro-diffusion method to analyse the $^{15}$N isotopic signature of organic N fractions in soil solutions or soil extracts (Chapter 5). In Chapter 6, this micro-diffusion method...
method is used to test whether the source, dynamics, and function of DON and CaCl$_2$ extractable organic N differ. The developed micro-diffusion method was also used in Chapter 7 to evaluate whether DON and EON are involved in N mineralization and how they interact with microbial biomass and the soil solids. Finally, the results of the previous chapters are synthesized in Chapter 8.

1.7. Definitions used in this thesis

Because of the huge variation in terminology among studies in the area of soil fertility, it is necessary to define the following terms as I use them in this thesis:

- **Soil N supply** or **potentially mineralizable N** refers to a bio-available fraction of soil organic matter that can mineralize in the long term (time scale 1-3 years). It is usually derived from long term laboratory incubations performed under controlled environmental conditions.

- **Mineralizable N** refers the amount of N in a soil that is actually released during a certain period (7 to 210 days). Mineralizable N can be determined in both the lab and the field, under aerobic and anaerobic conditions, but it is usually determined at a relatively short time scale (< 2 months).

- **Chemical (extraction) methods** or **soil N test** refer to those methods that are designed to extract a distinct organic matter fraction from soil, usually by shaking a soil with water or salt solutions. Processes responsible for the release of organic N during extraction include dissolution, desorption, and hydrolysis. The extracted organic matter fraction is abbreviated as EOM, whereas the extracted organic C and N fraction is abbreviated as EOC and EON, respectively.

- **Biological methods** refer to those methods that determine (potentially) mineralizable N by measuring increases of inorganic N over time.

- **Organic matter fraction** is used to describe measurable organic matter components. The organic matter fraction can be collected using chemical extraction or physical fractionation methods. It can be expressed as a percentage (e.g., % of total N) or as a concentration (mg kg$^{-1}$ or mg l$^{-1}$).
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Figure 1.1. Schematic diagram of the content of this thesis within the framework of my research regarding the predictive value of SOM fractions.

- **Organic matter pool** refers to a theoretically separated and kinetically delineated component of soil organic matter.

- **Labile, bio-available** or **biodegradable organic matter** consists of organic matter that is easily available and decomposable for microbes. It contrasts to **recalcitrant organic matter**, which consists of compounds that are less available or decomposable for microbes.
Chapter 1  Predicting soil N mineralization
CHAPTER 2

EXTRACTABLE AND DISSOLVED SOIL ORGANIC NITROGEN

A QUANTITATIVE ASSESSMENT

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Abstract

Extractable Organic N (EON) or Dissolved Organic Nitrogen (DON) pools are often analysed to predict N mineralisation, N leaching, and to evaluate agricultural (nutrient) management practices. Size and characteristics of both pools, however, are strongly influenced by methodology. Quantifying the influence of methodology can increase the accuracy of soil tests to predict N mineralisation, improve model simulations, and can help to quantify the contribution of the EON and DON pools to soil N cycling. We estimated the relative impact of methodological, management, and environmental factors on EON and DON, using a meta-analysis approach based on 127 studies. Our results indicate that the EON and DON pools are neither similar in size nor controlled by the same factors. The influence of factors controlling EON generally decreased in the order of methodology (10–2400%), followed by environment (11–270%) and management (16–77%). DON concentrations were primarily controlled by management factors: different land use and fertilisation caused a variation of 37–118%. Seasonal variations in DON concentrations were generally smaller than variations in EON, suggesting that high mineralisation and sorption rates buffer DON. The large range in EON as affected by different methodology emphasizes the importance of using appropriate and standardized methods for the determination of EON. The determination of DON can be useful to estimate leaching losses. EON, however, can be used to assess the impact of soil management practices on the turnover rate of labile soil organic matter pools.

2.1 Introduction

Awareness is growing that Dissolved Organic Nitrogen (DON) plays an important role in ecological processes such as N leaching, mineralisation, and plant uptake (Näsholm et al., 2000; Perakis & Hedin, 2002; Jones et al., 2004; Schimel & Bennett, 2004). It is also well known that a rapid rewetting of dry soils causes an increase in mineralisation and a pulse of available C and N (Fierer & Schimel, 2003). DON originates from plant litter leachates, microbial and root exudates, and hydrolysis of insoluble soil organic matter (Haynes, 2005). Dissolved organic N is defined as the fraction of soil organic nitrogen which is collected in situ using a lysimeter, rhizon or suction cup among other devices, and where no extractant is used. DON is defined as organic N present in dissolved form in soil solution (Murphy et al., 2000). In general, DON concentrations vary between 25 μg l⁻¹ and 10 mg l⁻¹ (Watson et al., 2000; Perakis & Hedin, 2002; Siemens & Kaupenjohann, 2002; Vinther et al., 2006), and account for 0.1 to 3.0% of soil total N (Haynes, 2005).

By contrast, Soluble Organic N (SON) or Extractable Organic N (EON) is soil N that is extracted from the soil using water, KCl, electro ultra-filtration (EUF), K₂SO₄, CaCl₂, or any other extractant (Murphy et al., 2000). Recently, Xiang et al. (2008) proposed that it would be more appropriate to call soil organic N which is obtained by extraction, EON rather than SON. The majority of results published on extractable organic N, which we used in this meta-analysis, used the term SON. Because the term EON is more appropriate, we use the term EON instead of SON.

The amount of EON in the soil can range from less than 5% of total N by mild salt solution (e.g. CaCl₂, diluted acids, etc.) to more than 50% by strong extraction methods such as acid hydrolysis (Stevenson, 1994; Matsumoto & Ae, 2004). Various EON pools are reported to relate with N mineralisation (Appel & Mengel, 1998; Mulvaney et al., 2001; Sharifi et al., 2007), land use changes, and agricultural management practices (Haynes, 2005). Typically, EON is extracted from field moist or dried soils by shaking with water or a salt solution at a high
soil-solution ratio for short periods of time, followed by centrifugation or filtering to separate the solution phase from the solid phase (Jones & Willet, 2006).

Conceptually, DON can be considered as a sub-pool of potentially EON that exists as a part of soil organic matter N. Tipping (1998) postulated that the EON pool “is a part of the soil solids and able to pass into solution under realistic soil conditions”. We consider EON as the sum of DON plus extra organic compounds that solubilise during extraction, originating from soil biomass and solid organic N (Fig. 2.1). The DON and EON pools are controlled and replenished by organic matter inputs (litter, manure), exudates, soil organic N, and influenced by adsorption–desorption and by microbial activity (Kalbitz et al., 2000). In soil, DON and EON pools are assumed to be in equilibrium (Qualls, 2000; Gjettermann et al., 2008). Size and characteristics of EON, however, depend strongly on how the EON pool is extracted (Stevenson, 1994; Matsumoto & Ae, 2004). There-

Figure 2.1. Methodological relationships between soil organic N, EON and DON: pool size of both DON and EON can vary due to differences in methodology used (denoted by black arrows).
Therefore, it is important to know the impact of methodology on the quantity and quality of EON and DON. An understanding of the impact of methodology on the size of the pools can lead to an increase in the accuracy of soil tests used to predict N mineralisation, to improve model simulations, and to quantify the contribution of DON to N leaching and plant N uptake.

Both DON and EON are mainly controlled by similar processes (Kalbitz et al., 2000; Qualls, 2000): mineralisation and immobilisation, adsorption and desorption, and precipitation and solubilisation. The release of DON rather than EON, however, is considered as a key controlling mechanism of terrestrial N cycling (Schimel & Bennett, 2004). We hypothesized that environmental parameters have a smaller impact on EON concentrations than on DON because of differences in size between the two pools (Fig. 2.1) and differences in chemical characteristics. Foremost, the extra solubilised compounds in EON are considered as organic N that potentially dissolves in time, having a lower turnover rate than DON (Tipping, 1998; Kalbitz et al., 2000; Zsolnay, 2003). Consequently, the concentration of DON is likely more affected by changes in microbial activity than EON. The turnover rate of DON is higher than that of EON, because EON is partly physically protected (Zsolnay, 2003).

DON is also enriched with labile hydrophilic compounds due to the selective sorption of recalcitrant, aromatic, and hydrophobic compounds from the soil solution (Guggenberger & Kaiser, 2003; Kalbitz et al., 2003). These hydrophilic compounds include small carboxylic acids, proteins, sugars, free amino acids and amino sugars (Qualls & Haines, 1991). However, these compounds comprise not only a range of moderately transformed plant-derived polysaccharides as well as microbial metabolites (Qualls & Haines, 1991), but also compounds of cell lysis (Christ & David, 1994). Drying soils before extraction, enhances the extraction of labile hydrophilic compounds (Kaiser et al., 2001), probably increasing the turnover rate of EON. In this situation, the impact of environmental parameters may have a bigger impact on EON than on DON, but it reflects a change in biomass rather than a change in an active soluble/dissolved pool of organic N. Changes in the size of the EON pool are also less apparent than a change in DON, because the size of the EON pool is generally larger than the active DON.
pool. Lastly, conditions that change the dynamics of the soil solution have a stronger effect on DON than on EON as part of the EON pool is adsorbed to the soil matrix. Conditions that change the dynamics of the soil solution include dilution after rainfall, transport, high ion concentrations after fertilisation, and inputs from the rhizosphere. In contrary to our hypothesis, it can be argued that following changes in environmental conditions or management practices, high mineralisation (Jones *et al*., 2004, 2005) and sorption rates (Kaiser & Zech, 1998) will reduce variations in the size of the DON pool. Consequently, measuring the pool size only may underestimate the size of the active DON pool. At present, however, it is not fully known whether EON and DON do react differently following a change in environment conditions or management practices.

As most studies on EON are focussed on statistical relationships with soil parameters (e.g. predicting N mineralisation) or human activities (e.g. soil management), a mechanistic understanding of this pool, and its interaction with DON, remains scant. This scant understanding is further hampered by methodological differences among studies (McDowell, 2003; Zsolnay, 2003). Variations in DON and EON pool size have been observed following a change in land use (Willet *et al*., 2004; Christou *et al*., 2005), seasonal variation (Leinweber *et al*., 1995; Jensen *et al*., 1997; Vinther *et al*., 2006; Weintraub & Schimel, 2005), drought and freezing (Schimel *et al*., 2007), drying and wetting cycles (Fierer & Schimel, 2002; Miller *et al*., 2005) and changes in management practices (Chantigny, 2003). Differences in soil characteristics also lead to differences in the size of the DON and EON pools (Kalbitz *et al*., 2000). The observed differences in the size of the EON pool following a change in land use, season, or management practices may be related to differences in the methodology used to determine the EON pool. However, studies on the size of DON and EON pools that separate the influence of methodology from environmental factors and management practices are scant.

We quantified the mean influence of methodology, environment and nutrient management on DON and EON levels, using the meta-analysis approach. This statistical technique reckons with methodological differences between studies and integrates independent data quantitatively (Gurevitch & Hedges, 1999, 2001).
We tested the hypothesis that DON concentrations are more prone to changes in environment and nutrient management than EON concentrations. More specifically, we addressed the following objectives:

- to determine how much of the variation in DON and EON concentrations is related to methodology versus management practices and environmental factors, and
- to assess whether methodology, management practices and environmental factors have a similar impact on DON and EON.

### 2.2 Data analysis

A meta-analysis can be used to estimate the average response of how DON and EON concentrations vary across a large number of studies due to a change in biophysical conditions or methodology, to test whether the change in biophysical conditions or methodology is significantly different from zero, and to examine the cause and effect of differences in DON and EON concentrations induced by changes in biophysical conditions or methodology. Data in a meta-analysis generally take the form of standardized metrics of an effect size and their associated sampling variances (Gurevitch & Hedges, 2001). We calculated the effect size in each experiment as the natural log of the response ratio ($R$, relative difference between 2 groups). The response ratio was calculated by dividing the mean of one group by the mean of a control group (Hedges et al., 1999; Rosenberg et al., 2000). For example, the influence of land use on DON levels was determined by calculating a relative difference between DON levels in arable, grassland and forest soils, using arable soils as control group. The mean difference between two groups among the analysed studies was calculated as described in Gurevitch & Hedges (2001).

Mean DON or EON levels of experimental and control groups with their standard deviations ($SDs$) and replicates ($n$), from a large number of studies were collected. A total of 200 studies published between 1980 and 2008 were identified, of which 127 studies included quantitative data for the control and treatment groups (reported in Appendix A.1). Data were subdivided in various
subgroups related to the factors that could affect the concentration of DON or EON (Table 2.1). To obtain sufficient data which would allow us to use the meta-analysis approach, studies which did not report SD or SE values were included in the analysis by using an arbitrary SD value based on a coefficient of variation (CV) of 1.5 times the average CV in the other studies (Alberton et al., 2005). Only studies which showed replication of the treatments were included. Error bars not identified were assumed to represent SE. If several values of the number of replicates were given, the lowest value was taken.

Table 2.1. Factors affecting DON or DON concentrations analyzed by meta-analysis. Subgroups are compared with control group for each of the listed factors

<table>
<thead>
<tr>
<th>Factors</th>
<th>Subgroups</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil drying</td>
<td>Soils that are analyzed field-moist, or dried (dried at 20, 40, or 105 °C)</td>
<td>Field-moist soils</td>
</tr>
<tr>
<td>Extraction T</td>
<td>Soils extracted at a temperature above 80 °C or at room temperature</td>
<td>Soils extracted at room temperature</td>
</tr>
<tr>
<td>Salt solution</td>
<td>Soils extracted with water, K₂SO₄, CaCl₂, Electro Ultra Filtration, or KCl</td>
<td>Soils extracted with CaCl₂ or KCl</td>
</tr>
<tr>
<td>Land use</td>
<td>Arable, grassland, and forest soils ✪</td>
<td>Arable soils</td>
</tr>
<tr>
<td>Fertiliser application</td>
<td>N fertiliser and no N fertiliser application</td>
<td>No N fertiliser application</td>
</tr>
<tr>
<td>Seasonality</td>
<td>Soils sampled in spring, summer, autumn or winter</td>
<td>Soils sampled in winter</td>
</tr>
<tr>
<td>Soil depth</td>
<td>Soils sampled in autumn/ winter, and soils sampled in spring/ summer</td>
<td>Soils sampled in autumn and winter</td>
</tr>
<tr>
<td>Soil pH</td>
<td>Soils with a pH &gt; 6 or &lt; 6</td>
<td>Soils with a pH &lt; 6</td>
</tr>
<tr>
<td>Soil texture</td>
<td>Clay, silt and sandy soils ✈</td>
<td>Sandy soils</td>
</tr>
<tr>
<td>Total soil N content</td>
<td>Soils with total N content &lt; 2 g kg⁻¹, between 2 and 4 g kg⁻¹, or &gt; 4 g kg⁻¹</td>
<td>Soils with total N content &lt; 2 g kg⁻¹</td>
</tr>
</tbody>
</table>

* Litter horizons of forest soils are excluded from the analysis

† The sandy group included texture class sand and all sandy soils; the silt group included silt and all silty soils; the clay group included all soils with at least 50% clay. Clay loam soils were used in both the silt and clay groups whereas loam soils were used in both the sand and silt groups.
The mean difference between two groups is significantly different from zero if both the upper and lower confidence limits were positive or negative. When the pooled within-class variance ($\sigma^2_{\text{pooled}}$) was higher than zero, a random effect model was used, whereas a fixed effect model was used when that quantity was equal to or smaller than zero. Means of response variables of different subgroups and differences between DON and EON were tested for significant differences based on the model heterogeneity test ($Q$-test), which is tested against a chi-square distribution as implemented in MetaWin ($P < 0.05$).

When insufficient quantitative data were available for the meta-analysis, we reviewed the influence of methodology on concentrations of EON and DON using results from individual studies. Methodological changes in the quality of DON and EON were assessed using their influence on the contribution of labile compounds to the total amount of DON and EON. Relevant observations from research on Dissolved Organic C or Extractable Organic C pools were also included in this analysis.

### 2.3 Results

#### 2.3.1 Influence of methodology on DON and EON

Most of the variations in extraction method significantly affected levels of EON (Table 2.2, Fig. 2.2). The EON levels increased with increasing drying temperature prior to extraction, extraction temperature ($T$), molarity of salt solutions, and soil-solution ratios. Centrifugation, dilution, and filtration decreased the amount of EON. The mean difference between dried and field-moist soil, hot and cold extraction temperatures, and among several weak salt solutions was quantified (Fig. 2.2).

Foremost, drying soil samples at 20 °C prior to extraction caused an average increase in EON of 245%. The increase was related to the drying temperature ($P < 0.001$); soils dried at 20, 40 or 105 °C showed an average increase of EON of 245, 400, and 2400%, respectively. Second, extraction of soil samples with water resulted in significantly lower contents of EON than when 0.01 M CaCl$_2$ or 1–2 M KCl was used (Fig. 2.2). Comparing other extraction methods,
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electro ultra-filtration (EUF) and NaHCO₃ extraction resulted in significantly higher EON levels than when CaCl₂ was used, whereas K₂SO₄ extracted lower amounts of EON than CaCl₂. Finally, hot extractions (80 to 100 °C) resulted in significantly higher amounts of EON than cold extractions (20 °C) with an average increase of 147% (Fig. 2.2). Looking at the individual extraction methods, T showed a significant effect with an average increase of 95% when CaCl₂ was used, 261% for water and 47% for EUF. Although the influence of methodology on the characteristics of EON was often unclear, its stability decreased with an increase in the drying T, and an increase in the soil-solution ratios (Table 2.2).

2.3.2 Influence of management practices on DON and EON

Levels of DON and EON were affected by land use (Fig. 2.3). Compared to arable soils, average DON concentrations were lower (~35%) in forest soils and grassland soils (~64%). In contrast, average EON content was higher in grass-

![Figure 2.2. Average change in % of the concentration of EON due to soil drying, extraction solution temperature, and salt solution. For groupings, see table 2.1. Error bars denote 95% confidence interval. *N = number of observations.](image)

32
land soils (+81%) than in arable soils. Compared to arable soils, forest and grassland soils differed significantly in DON and EON levels ($P < 0.01$). A maximum increase of 118% was found in EON levels following the application of inorganic and organic fertilisers (Fig. 2.3). DON increased with 57% following the application of inorganic N fertilisers whereas EON increased with 17%. Incorporation of crop residues or manure increased EON with 22% and 70%, respectively.

2.3.2 Influence of environmental factors on DON and EON
DON concentration measured in spring/summer did not differ significantly from autumn/winter measurements (Fig. 2.4). Average EON concentrations, however, were significantly higher in spring/summer (+56%) compared to the autumn/winter period. When individual seasons were compared, significant differences in EON concentrations between the winter and autumn (+110%), and between

<table>
<thead>
<tr>
<th>Activity</th>
<th>Variation</th>
<th>Quantity a</th>
<th>Stability a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Storage temperature soil (°C)</td>
<td>–20 to 70</td>
<td>–/+</td>
<td>0/+</td>
</tr>
<tr>
<td>Drying temperature soil (°C)</td>
<td>20 to 105</td>
<td>++</td>
<td>–/+</td>
</tr>
<tr>
<td>Drying soils (–)</td>
<td>Yes/ No</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>Sieving soil (mm)</td>
<td>&lt;1 to 10</td>
<td>0/+</td>
<td>?</td>
</tr>
<tr>
<td>Extractant (–)</td>
<td>weak – strong salts</td>
<td>–/+</td>
<td>–/+</td>
</tr>
<tr>
<td>pH of extractant (–)</td>
<td>2 to 8</td>
<td>–/+</td>
<td>–</td>
</tr>
<tr>
<td>Soil–solution–ratio (g.l⁻¹)</td>
<td>25 to 500</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Extraction temperature (°C)</td>
<td>5 to 120</td>
<td>++</td>
<td>–/+</td>
</tr>
<tr>
<td>Extraction time (min)</td>
<td>5 to 1440</td>
<td>++</td>
<td>?</td>
</tr>
<tr>
<td>Dilution (–)</td>
<td>1 to 10</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Filtration (um)</td>
<td>0.1 to 0.45</td>
<td>–</td>
<td>0/+</td>
</tr>
<tr>
<td>Centrifugation (–)</td>
<td>Yes/ No</td>
<td>–/+</td>
<td>?</td>
</tr>
<tr>
<td>Centrifugation time (min)</td>
<td>5 to 60</td>
<td>–/+</td>
<td>?</td>
</tr>
<tr>
<td>Centrifugation force (g)</td>
<td>5.000 to 20.000</td>
<td>–</td>
<td>?</td>
</tr>
<tr>
<td>Storage soil solution (°C)</td>
<td>–20 to 20</td>
<td>–/+</td>
<td>0/+</td>
</tr>
</tbody>
</table>

* ++ strong positive influence; + positive influence; 0 no clear trend; - negative influence; ? Unknown influence
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Winter and summer (+268%) were found. Levels of DON and EON decreased with depth, with an average difference of 36–49% between the topsoil and subsoil. Decreases in DON and EON concentrations by depth were almost similar (36 and 49%; \( P = 0.043 \)). Whereas an increase in pH (pH > 6) led to significantly lower EON concentrations (−22%), it caused DON concentrations to become highly variable (Fig. 2.4). Overall, the response of DON and EON to soil pH was significantly different (\( P = 0.012 \)). These results agreed with the effect on liming, which showed a tendency (\( P > 0.05 \)) to decrease EON and increase DON concentrations (Fig. 2.3).

The concentration of EON was positively related to total soil N (Fig. 2.4). Compared to soils with an N content of smaller than 2 g kg\(^{-1}\) the EON levels increased by 240% for soils when the total N content was higher than 4 g kg\(^{-1}\). For

![Figure 2.3](image-url)
soils with a total soil N content between 2 and 4 g kg\(^{-1}\) the EON concentration increased by 61%. The EON content in non-sandy soils was 40–50% higher than in sandy soils. EON content did not differ significantly between silt and clay soils (\(P = 0.698\)). Due to lack of sufficient data, the influence of total N and texture on DON concentrations could not be quantified.

### 2.4 Discussion

#### 2.4.1 Influence of methodology on EON contents

The most important factor that causes the soil EON content to change is the method which is used to extract EON. Depending on which method is used, it could change the EON content by 2400% (Fig. 2.2). The significant increase in EON due to an increase in drying T prior to analysis (Fig. 2.2; Table 2.2) likely
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originates from lysis of microbial biomass following desiccation and from disrup-
ted organic-mineral associations and subsequent release of organic com-
pounds (Haynes et al., 1991; Appel et al., 1996; Appel & Mengel, 1998; Haynes,
2005). Similarly, an increase in extraction T, the molarity of salt solutions, and
soil-solution ratios increases EON contents (Kaiser et al., 2001; Matsumoto &
Ae, 2004; Fest et al., 2008) due to their effect on sorption equilibria. Centrifuga-
tion, dilution, and filtration however, decrease the amount of EON by floccula-
tion or adsorption, depending on the filter size used, centrifugal force, pH and
the initial characteristics of the particulate organic matter (POM) fractions
(Table 2.2; You et al., 1999; Rees & Parker, 2005). POM fractions consist of or-
ganic particles that are not bound to mineral particles (Haynes, 2005; Gregorich
et al., 2006). POM is an intermediate between plant residues and soil organic
matter (Gregorich et al., 2006) and interacts with DOM. For example, Zsolnay
(2003) illustrated how DOM could be transformed into POM during filtration
because of changes in its tertiary structure. Unfortunately, the size of the data-
base was too limited to better quantify the influence of all methodological differ-
ences on EON.

Methodological differences are also known to influence the size of the bio-
logically active part of the EON pool (~stability; Table 2.2). The increase in EON
contents following drying the soil prior to extraction mostly consists of easy de-
gradable compounds (Appel & Mengel, 1990, 1993; Nunan et al., 2001). Drying
soils prior to extraction is therefore likely to result in an increase in the biologi-
cally active EON pool. Decreasing stability of EON is also related to increasing
soil-solution ratios (Kaiser et al., 2001), filtration (You et al., 1999; Rees & Par-
kerr, 2005) and the use of specific salt solutions (Rennert et al., 2007). Calcium,
for example, is able to reduce the solubility of high molecular weight compounds
(Römkens & Dolfing, 1998; Reemtsma et al., 1999; Rennert et al., 2007). Conse-
quently, the relative contribution of smaller compounds will increase. High ex-
traction temperatures (>80 °C) and use of stronger salt solutions however, in-
crease the solubilisation of recalcitrant compounds and are subsequently related
to an increase in stability of EON (Table 2.2; Michrina et al., 1982; Matsumoto &
Ae, 2004). Because the biochemical characteristics of EON affect its contribution
to N mineralisation and sorption, qualitative information about the EON pool remains important.

Extraction conditions and prior sample treatment strongly affect whether more or less microbial N, physically protected, chemically adsorbed or solid organic N is solubilised and extracted. This will definitely impact the role of the analysed EON in soil N cycling, and most likely also the value of EON as an indicator for bioavailable N. But how and to what extent, is not known, yet. Accounting for this impact is necessary in increasing our understanding of the role of EON in soil N cycling. The strong impact of methodology also emphasizes the importance of clearly describing the methodology used to obtain EON. There is a strong need of consistency when the methodology is been developed for the relationship between extractable soil N and plant N uptake (Wang et al., 2001; Griffin, 2008). It is also clear that when the extractable N is used to make fertiliser-N recommendations, the extraction methodology used to develop the above described relationship is closely followed. Methodology is known to affect the chemistry of the soil solution (Ludwig et al., 1999; Geibe et al., 2006; Weihermüller et al., 2007), and is therefore also likely to affect DON concentrations. For example, the amount and characteristics of DOM vary among pore size classes (Zsolnay, 2003) and consequently, DON concentrations are affected by the centrifugal force or amount of suction applied to collect the soil solution. In addition, specific sorption and contamination from solvents and flexibilisers may induce potential artefacts (Weihermüller et al., 2007). Until now, there is still a need to perform a quantitative and qualitative comparison of common devices used to collect DON.

2.4.2 Responses of DON and EON to management and environment

In general, changes in land use and nutrient management caused a different response of the size of the DON and EON pools (Fig. 2.3). Likewise, changes in the DON and EON concentrations induced by season, soil depth or pH were different (Fig. 2.4). As both pools are mainly controlled by immobilization, mineralisation, sorption and desorption (Kalbitz et al., 2000; Qualls, 2000), a different response implies that the relative impact of these processes differ for both pools.
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Predicting soil N mineralization

Impact of land use and fertilisation on DON and EON

Larger variations in DON compared to EON were only observed as a response to inorganic N fertilisation and when forest soil was compared with arable soils. Following the application of inorganic N-fertiliser it is known that the concentration of DON increases, as the application of inorganic N-fertiliser solubilises soil organic N because of its pH effect (Chantigny, 2003). The addition of inorganic N-fertiliser changes the microbial community structure, and increases the hydrophilic character of DON due to an increased production of N-rich fractions or a decrease in consumption (McDowell et al., 2004). Although the variation in the absolute concentration of EON is larger than that for DON (data not shown), the relative increase in EON content following the application of fertiliser-N is only 10% whereas for DON the increase was 50% (Fig. 2.3). DON concentrations were lower in forest soils than that in arable soils, whereas concentrations of EON tend to be higher in forest soils. Dissolved organic C and extractable organic C levels were also significantly higher in forest than arable soils (data not shown). Higher levels of C often contribute to a lower biodegradability of organic compounds: forest litter contains higher lignin content and C to N ratio than agricultural crop residues (Chantigny, 2003). Lower DON levels in forest and grassland soils, therefore, could be the result of higher immobilization rates or sorption: more N is needed for decomposition of C rich compounds and these compounds show also a stronger sorption affinity (Guggenberger & Kaiser, 2003).

High release of root exudates in grassland soils is likely to increase the rate of immobilization of DON compared to arable soils, leading to a decrease in the concentration of DON (Khalid et al., 2007). This agrees with our observation that the average DOC concentrations were higher in grassland soils than arable soils (data not shown). In contrast to DON, the concentrations of EON were higher in grassland soils than in arable soils which may be attributed to their higher organic matter and biomass content (Haynes, 2000). Because most measurements of EON are performed on dried soils, higher EON contents in grassland soils partly reflect a higher contribution of lysed microbial cells.
**Seasonality**

The most important factors controlling DON leaching losses are likely related to water fluxes through the soil profile and without significant precipitation or irrigation, leaching will not occur (Van Kessel *et al*., 2009). Rewetting and drying of soils which are common occurrences in arid, semi-arid, or Mediterranean-type environments, have short term but pronounced effects on the content of soluble organic compounds and N mineralisation in surface and subsurface soil (Franzluebbers, 1999; Fierer & Schimel, 2002; Xiang *et al*., 2008). Increasing the frequency of wetting and drying and adding Adenostoma litter had no effect of CO₂ release but significantly increased DON concentrations (Miller *et al*., 2005). Although DON concentrations are related to the contact time between the soil and the soil solution (McDowell & Wood, 1984; Michalzik & Matzner, 1999), suggesting seasonal variation in DON concentrations, we observed no significant influence of different seasons on DON concentrations (Fig. 2.4).

In contrast, EON concentrations were significantly different between summer and winter periods, and between autumn and winter periods. Changes in seasons lead to differences in temperature and moisture which are important factors controlling microbial activity and its adaptation (Schmidt *et al*., 2007). An absence of a significant change in the DON concentration would suggest that microbial assimilation and/or the rate of mineralisation caused the concentrations of DON to remain low. The DON pool is often characterised to be highly dynamic with a high turnover rate (<4 h) (Kaiser & Zech, 1998; Jones *et al*., 2005; Van Hees *et al*., 2005). It is possible that differences in the size of the DON pool following a change in season cannot be detected when measured at weekly or monthly intervals. As abiotic (sorption equilibria) and biotic (microbial uptake and release) processes control the concentration of DON, the most pronounced change in the DON concentration is likely to occur in the surface layer and not in the subsoil. As seasonal measurements of DON, however, are often determined below the cultivated layer which show lower DON concentrations (Van Kessel *et al*., 2009), seasonal variations in DON concentrations will be smaller.
Chapter 2  Predicting soil N mineralization

Soil characteristics
Because the majority of the organic matter input occurs in the plough layer, the topsoil layer shows the highest soil organic matter content and microbial activity which will decrease with depth. Likewise, the concentrations of EON and DON are the highest in the topsoil and decrease by depth (Fig. 2.4). Although the difference between DON and EON due to depth is significant \((P = 0.04)\), the different response to depth is small (about 10%). The decrease in the EON concentration as affected by an increase in soil pH (Figs. 2.3 and 2.4) may be explained by its increasing solubility (Andersson \textit{et al.}, 1994), increased microbial activity and consumption of soluble molecules (Karlik, 1995), or an increased sorption by cation bridging due to high Calcium concentrations (Römkens & Dolfing, 1998). Although decreases in the contents of EON are likely to cause higher DON concentrations, the dataset was not large enough to be able to draw a firm conclusion. The influence of soil pH on DON is therefore less clear. If similar processes affect DOC and DON concentrations, the effect of pH would be small within the normal pH range as they occur in agricultural soils (Kalbitz \textit{et al.}, 2000). Levels of DON are likely to increase with increasing total soil N and a decrease in clay content (Kalbitz \textit{et al.}, 2000), but the meta-analysis approach could not be used to quantify this increase.

2.5 Concluding remarks
Although a mechanistic understanding of the functions of EON remains limited, its usefulness as indicator of how agricultural management strategies affect soil N dynamics has been proven. Significant variation in EON as affected by total soil N content, soil pH, fertilisation, and land use supports this observation. Because of substantial seasonal variability, it emphasizes the need that sampling be carried out at the same time each year in order to make comparison possible. Otherwise, temporal variation may obscure differences due to changes in management practices. Even more important is the impact that methodology can have on the concentration of EON. Accounting for this impact is necessary to
make progress in increasing our mechanistic understanding of the role of EON in soil N cycling.

Compared to DON, the content of EON was more prone to changes in seasons, soil depth, pH, and cropping systems. These findings invalidate our hypothesis that variations in the concentrations of DON are more pronounced than variations in EON contents. They support the suggestion that the flux of organic compounds through both the DON and the EON pools is affected by changes in biophysical–environmental conditions and management practices. When the system is not at steady state (e.g. in response to N-fertiliser), changes in the N flux through the DON and EON pools will cause a change in their characteristics, because both sorption and mineralisation are selective for specific compounds. Qualitative information of both the DON and the EON pools, therefore, will also be useful to understand their role in the soil N cycle.

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CHAPTER 3

NITROGEN MINERALIZATION

A REVIEW AND META-ANALYSIS OF THE PREDICTIVE VALUE OF SOIL TESTS

Gerard H. Ros
Erwin J.M. Temminghoff
Ellis Hoffland
Abstract

Accurate estimation of mineralizable nitrogen (N) from soil organic matter is essential to improve fertilizer management in agricultural systems. Mineralizable N refers to the amount of N in soil that is released during a certain period (ranging from 1 week to the length of a growing season). It has been estimated from increases in inorganic N during incubation or from N uptake by plants grown in a greenhouse or field. Many chemical soil tests measuring extractable organic N (EON) fractions have been proposed to predict mineralizable N. We evaluated the predictive value of these soil tests, using 2068 observations from 218 papers. Meta-analysis was used to find the best soil test, to analyse differences between field and laboratory experiments, and to determine whether their predictive value is affected by extraction intensity (% of total soil N that is extracted). The concentration of EON was positively related to mineralizable N, explaining on average 47% of the variation. It did not, however, explain more of the variation than total N. Best predictions (57% < R² < 74%) were obtained when EON was extracted with hot CaCl₂, acid KMnO₄, acid K₂Cr₂O₇, hot water or hot KCl. Extraction intensity was not related to the strength of the above-mentioned relationship. Predictions of mineralizable N were significantly worse when mineralization was measured in the field compared with measurements under controlled conditions. We found no evidence of a causal and direct relationship between EON and mineralizable N. Accuracy of soil testing may improve when the current ‘single soil test approach’ changes to a more complex approach, which includes soil properties and environmental conditions.

3.1 Introduction

Intensive agricultural production has resulted in large nitrogen (N) losses to the environment. In particular, leaching of nitrate (NO$_3^-$) and emission of NH$_3$ and N$_2$O have an impact on the quality of the environment (Hansen et al., 2001; Velthof et al., 2009). One way to reduce these losses is to increase N-use efficiency by using sustainable management practices (Wivstad et al., 2005) that, for example, match plant-available N to plant demand. Sustainable N management requires a thorough assessment of the N supply from soil and organic amendments.

Since 1900, soil N supply has been estimated using biological and chemical methods, including tests for available nitrate and (potentially) mineralizable N (Keeney, 1982). Mineralizable N refers to the amount of N in soil that is released during a defined period, and is usually expressed in mg kg$^{-1}$ or as a rate (mg kg$^{-1}$ day$^{-1}$). The experimental period during which mineralization is measured ranges from 7 to 210 days (Keeney, 1982). Potentially mineralizable N refers to the amount of N that mineralizes under optimum and constant environmental conditions, and is usually derived by fitting a first-order kinetic model to inorganic N concentrations over time (Stanford & Smith, 1972).

Biological methods estimate mineralizable N from gross or net increases in inorganic N during incubation (Wang et al., 2001), from potential pools or rates calculated from long-term incubation (Sharifi et al., 2007a), or from N uptake by plants grown in greenhouse or field experiments (Fox & Piekielek 1984). These biological methods are considered to be the most reliable estimators but they are expensive, time-consuming and labour-intensive and their results strongly depend on experimental conditions (Keeney, 1982). Therefore, chemical methods have been proposed as an alternative, such as extraction with hot KCl (Gianello & Bremner, 1986a) or 0.01 M CaCl$_2$ (Appel & Mengel, 1998), or using the Illinois soil N test (ISNT) (Williams et al., 2007).

Chemical methods are used to extract a N fraction that relates statistically to (potentially) mineralizable N determined by biological methods. Most of
these methods were developed before 1980 and since then there have been few developments; papers published during the past decades evaluate existing soil tests or slight modifications of them (Bremner, 1982; Griffin, 2008).

Chemical methods measure NH$_4^+$ or organic N in extractants such as water and hydrolysing salt solutions (Haynes, 2005). The amount of (hydrolyzed) organic N in the extractant can range from less than 5% to more than 50% of total N depending on the intensity of the extraction (Matsumoto & Ae, 2004). The extraction intensity determines how much organic N is released from the soil. It primarily depends on salt type, the molarity of the solution, the soil-to-solution ratio, and the duration and temperature of extraction (Ros et al., 2009).

Extractable organic N (EON) has been shown to correlate significantly with (potentially) mineralizable N (Gianello & Bremner, 1986a; Appel & Mengel, 1998). This relationship has been explained by two causal mechanisms: EON is assumed to be a source of mineralized N or an indicator of microbial activity. Schimel & Bennett (2004) proposed that dissolved organic nitrogen (DON), which is a component of EON, plays an intermediate role in N mineralization, and proposed that the flow of N through the DON pool, rather than its size, controls the rate of mineralization. It can be questioned whether this transient DON pool can be assessed accurately by current soil tests, because most soil tests extract also organic compounds other than DON (Kelley & Stevenson, 1985; Von Lützow et al., 2007; Ros et al., 2009), and DON itself can be separated into active and non-active fractions (Appel & Xu, 1995; Jones et al., 2004).

EON may also relate to the size of microbial biomass. The size of the biomass is assumed to be proportional to mineralizable N (Jenkinson, 1968; Booth et al., 2005), so the best chemical methods may consist of those that selectively extract biomass N. This is probably why milder extractants have been favoured rather than more intensive ones or total N, as a larger proportion of EON in milder extractions originates from microbial biomass (Kelley & Stevenson, 1985). However, a relationship between extraction intensity and predictive value of soil tests has not been tested for so far.
Predicting N mineralization: a meta-analysis

Introduction of a single EON fraction in fertilizer recommendations is only useful when the slope of the regression between EON and mineralizable N is independent of other variables such as year, experimental site and management history. However, numerous papers have shown that such slopes differ between seasons (Last & Draycott, 1971; Sharifi et al., 2007b), soil texture and pH classes (Stanford & Smith, 1978; Groot & Houba, 1995), and land uses, drainage classes and soil management histories (Verstraeten et al., 1970; Fox & Piekielek, 1984; Williams et al., 2007). Hence, the assumption that a single EON fraction, without these covariates, can be used to improve fertilizer recommendations is open to criticism.

Comparison and evaluation of soil tests are strongly hampered by variation in methodology. Methodological issues affecting the relationship between EON and mineralizable N include both the experimental design to assess mineralizable N and the extraction procedure. Issues related to the experimental design include the duration of the experiment, the soil water potential and the temperature during the experiment. Important characteristics of the extraction procedure are, among others, the temperature used to dry the soils prior to extraction, the duration and temperature of the extraction, and the salt solution used (Ros et al., 2009). Another complicating issue is the variation in statistical analysis and experimental design, in particular with regard to the quality of the regression, including the number of included soils, presence of homoscedasticity and influential points. Use of meta-analysis may be helpful in quantifying the confounding influence of methodology on the relationship of EON with mineralizable N (Gurevitch & Hedges, 2001). This statistical technique takes account of methodological differences between studies and integrates independent data quantitatively. Until now, and as far as we are aware, meta-analysis has not been used for comparison of soil tests.

The aim of this study is to find the best chemical methods predicting N mineralization that have been proposed and evaluated during the last 100 years. Our evaluation encompasses both the strength and the slope of the relationship between EON and (potentially) mineralizable N. More specifically, we focus on the following hypotheses that (i) all EON fractions are positively related to
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(potentially) mineralizable N, (ii) (potentially) mineralizable N is more strongly correlated with EON than with total N, (iii) the relationship between EON and (potentially) mineralizable N is negatively related to the intensity of extraction, (iv) the relationship between EON and (potentially) mineralizable N is improved when mineralizable N is measured under controlled conditions (laboratory, greenhouse) rather than in field experiments and (v) the slope of the above-mentioned relationship depends strongly on the characteristics of individual studies.

3.2 Data analysis

3.2.1 Data collection

Data were collected on: the correlation coefficients between EON and total N on the one hand, and (potentially) mineralizable N on the other; the number of soils on which the correlation was based; and the main aspects of methodological design, including incubation temperature, duration and extraction conditions. We collected 2068 correlations presented in 218 papers (of which 211 were in peer-reviewed journals; see Appendix A2) in which mineralizable N was estimated using net or gross increases in inorganic N (n = 988), N uptake (n = 549), fitted potential mineralizable N pools or mineralization rates (n = 162), or economic optimum N rates (n = 27). Most experiments were performed in arable ecosystems in the northern hemisphere, in particular in the developed countries. Experiments were performed in laboratory (n = 1013), greenhouse (n = 686) and field (n = 369). We excluded soil tests that included NO$_3$ in their analysis of extractable N. This was because the correlation between extracted and mineralizable N may be confounded by NO$_3$ because it shows strong temporal variation, and hence, its inclusion can only be rationalized when all soils have been under similar climatic conditions and management practices and collected at the same time (Wang et al., 2001). We evaluated those soil tests that were tested in at least five studies during the last 100 years. These soil tests included the use of acid and alkaline KMnO$_4$, Ba(OH)$_2$, Ca(OH)$_2$, NaOH, CaCl$_2$, KCl, K$_2$SO$_4$, Electro
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- Ultra-filtration (EUF), \( \text{H}_2\text{SO}_4 \), HCl, water, NaHCO\(_3\), phosphate buffer and acid \( \text{K}_2\text{Cr}_2\text{O}_7 \). Information on extraction procedures is given in Table 3.1.

### 3.2.2 Meta-analysis correlations

Meta-analysis was performed on the total dataset to quantify mean correlation coefficients for each soil, independently of how mineralization was measured. In addition, it was performed separately for laboratory, greenhouse and field measurements. Laboratory experiments included aerobic incubations with \( n = 322 \) or without leaching \( n = 315 \) and anaerobic incubations \( n = 376 \). The mean correlation coefficient for each soil test was calculated by fixed-effect or random-effect models. A random-effect model was used if the pooled within-class variance was greater than zero, and a fixed-effect model was used if the variance was equal to or less than zero (Ros et al., 2009). A quantitative index of the

Table 3.1. Characteristics of chemical extraction methods used in the papers analysed; observed range in molarity (M), soil solution ratios (SSR), extraction temperature (Temp), extraction time (Time) and measured \( N \) fraction for 18 soil tests.

<table>
<thead>
<tr>
<th>Chemical methods</th>
<th>Characteristics</th>
<th>Method *</th>
<th>Salt(s) *</th>
<th>M (mol l(^{-1}))</th>
<th>SSR (ml g(^{-1}))</th>
<th>Temp (°C)</th>
<th>Time (min)</th>
<th>N-fraction (^{+})</th>
<th>n *</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUF</td>
<td></td>
<td>Hot CaCl</td>
<td>CaCl(_2)</td>
<td>0.01</td>
<td>5 – 10</td>
<td>20 – 25</td>
<td>30 – 120</td>
<td>1 + 2</td>
<td>16</td>
</tr>
<tr>
<td>Cold CaCl</td>
<td></td>
<td>NaOH</td>
<td>NaOH</td>
<td>0.1 – 10</td>
<td>2 – 10</td>
<td>20 – 25</td>
<td>50 – 120</td>
<td>1 + 2</td>
<td>2</td>
</tr>
<tr>
<td>Hot KCl</td>
<td></td>
<td>KCl</td>
<td>KCl</td>
<td>1 – 2</td>
<td>2 – 10</td>
<td>20 – 25</td>
<td>60 – 240</td>
<td>1 + 2</td>
<td>8</td>
</tr>
<tr>
<td>Hot KCl</td>
<td></td>
<td>KCl</td>
<td>KCl</td>
<td>1 – 3</td>
<td>2 – 10</td>
<td>35 – 120</td>
<td>60 – 120</td>
<td>1 + 2</td>
<td>51</td>
</tr>
<tr>
<td>Cold water</td>
<td></td>
<td>-</td>
<td>-</td>
<td>2 – 3</td>
<td>20 – 25</td>
<td>15 – 30</td>
<td>30 – 90</td>
<td>1 + 2</td>
<td>3</td>
</tr>
<tr>
<td>Hot water</td>
<td></td>
<td>-</td>
<td>-</td>
<td>2 – 8</td>
<td>80 – 100</td>
<td>60 – 990</td>
<td>1 + 2</td>
<td>35 – 80</td>
<td>16</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td></td>
<td>-</td>
<td>-</td>
<td>4 – 10</td>
<td>100 – 120</td>
<td>100 – 120</td>
<td>1 + 2</td>
<td>100 – 120</td>
<td>24</td>
</tr>
<tr>
<td>Acid ( \text{K}_2\text{Cr}_2\text{O}_7 )</td>
<td></td>
<td>-</td>
<td>-</td>
<td>0.02 – 1.0</td>
<td>1 – 60</td>
<td>20 – 25</td>
<td>30 – 60</td>
<td>1 + 2</td>
<td>8</td>
</tr>
<tr>
<td>NaOH</td>
<td></td>
<td>-</td>
<td>-</td>
<td>0.1 – 10</td>
<td>2 – 10</td>
<td>20 – 100</td>
<td>4 – 2520</td>
<td>1 + 2</td>
<td>27</td>
</tr>
<tr>
<td>BaOH(_2)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>0.05 – 0.1</td>
<td>10</td>
<td>20 – 25</td>
<td>30 – 90</td>
<td>1 + 2</td>
<td>14</td>
</tr>
<tr>
<td>CaOH(_2)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>0.05</td>
<td>10</td>
<td>100</td>
<td>30</td>
<td>1 + 2</td>
<td>6</td>
</tr>
<tr>
<td>Alkaline ( \text{KMnO}_4 )</td>
<td></td>
<td>-</td>
<td>-</td>
<td>0.01 – 0.1</td>
<td>5 – 150</td>
<td>100</td>
<td>5 – 30</td>
<td>1 + 2</td>
<td>41</td>
</tr>
<tr>
<td>Acid ( \text{KMnO}_4 )</td>
<td></td>
<td>-</td>
<td>-</td>
<td>0.01 – 0.1</td>
<td>1 – 50</td>
<td>20 – 25</td>
<td>60 – 120</td>
<td>1 + 2</td>
<td>14</td>
</tr>
<tr>
<td>NaHCO(_3)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>0.01 – 0.5</td>
<td>5 – 20</td>
<td>20 – 25</td>
<td>15 – 300</td>
<td>2</td>
<td>28</td>
</tr>
<tr>
<td>HCl</td>
<td></td>
<td>-</td>
<td>-</td>
<td>0.02 – 8</td>
<td>1 – 10</td>
<td>20 – 115</td>
<td>30 – 1440</td>
<td>1 + 2</td>
<td>16</td>
</tr>
<tr>
<td>H(_2)\text{SO}_4</td>
<td></td>
<td>-</td>
<td>-</td>
<td>0.1 – 1.5</td>
<td>2 – 80</td>
<td>20 – 100</td>
<td>15 – 1680</td>
<td>1 + 2</td>
<td>23</td>
</tr>
<tr>
<td>K(_2)\text{SO}_4</td>
<td></td>
<td>-</td>
<td>-</td>
<td>0.5 – 1</td>
<td>2 – 10</td>
<td>20 – 100</td>
<td>30 – 720</td>
<td>2</td>
<td>7</td>
</tr>
</tbody>
</table>

* EUF = electro-ultrafiltration; soil test abbreviations consist of the salt used to extract an organic N fraction; hot and cold refers to temperature of extraction: hot (> 80 °C) and cold (< 25 °C)

+ Determination EON included the analysis of NH\(_4\) (1) or excluded the analysis of NH\(_3\) (2)

\( n \) is the number of studies evaluating the particular soil test
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strength of relationship in each experiment was calculated from Fisher’s r-to-z transformation (Cooper, 1998). Mean z-values for all observations and groups were calculated as described in Gurevitch & Hedges (2001). After calculation of mean z-values, z-values were back-transformed to r-values by Fisher’s z-to-r transformation. Mean z-values of different groups (for example EON pools, total N) were tested for significant differences based on the model heterogeneity test, which is tested against a $\chi^2$ distribution (Rosenberg et al., 2000).

Publication bias (under-reporting of experiments without significant results) can lead to an over-estimation of the strength of the relationship. The presence of publication bias was tested using the rank correlation tests of Kendall and Spearman (Rosenberg et al., 2000). We also calculated fail-safe numbers as suggested by Rosenthal (1979). A fail-safe number is the number of non-significant, unpublished or missing studies that would need to be added to a meta-analysis in order to change the result of the meta-analysis from significant to non-significant. If this number is large (>5 × n + 10) relative to the number of observed studies (n) (Gurevitch & Hedges, 2001), there is confidence that the observed result, even with some publication bias, is a reliable estimate of the strength of the relationship.

3.2.3 Meta-analysis of regression lines
Slopes of the relationship between EON (mg kg$^{-1}$) and mineralizable N (mg kg$^{-1}$ day$^{-1}$) were estimated using data presented. If data were presented graphically, slopes were estimated from figures after digitization using Plot Digitizer Version 1.9 (M. Boleman, Department of Physics, University of South Alabama, Mobile, Alabama, USA). Regression slopes were synthesized for each soil test across all experiments, on the basis of a weighted least squares approach (Becker & Wu, 2007). If we denote the individual estimates of the regression slopes as $a_1, a_2, \ldots$, $a_i$, we can compute the combined slope $\alpha$ as:

$$\alpha = \frac{\sum_{i=1}^{k} w_i a_i}{\sum_{i=1}^{k} w_i}$$

3.1.
where \( n \) is the number of slopes combined, \( a_i \) is the slope from study \( i \) and \( w_i \) is the weight for that slope in the \( i \)th study, which is the reciprocal of the slope variance. Minimum, maximum and combined slope were reported for each soil test.

### 3.2.4 Detailed analyses for hot KCl

We additionally investigated the influence of methodology on the strength of the relationship between hot-KCl-extractable organic N and mineralizable N. This included treatment prior to the start of the experiment (drying soils) and extraction characteristics (variation in temperature, soil solution ratios and duration). Their influence on individual regression slopes was analysed using linear regression. The slopes used (\( n = 104 \)) were log-transformed to get a normally distributed dataset. Samples were divided into four groups related to experimental type regarding how N mineralization was measured (anaerobic incubation, aerobic incubation, field and pot experiments), into two groups related to the sample pre-treatment (dried and field-moist), and into two groups related to the extraction conditions (either following the procedure of Gianello & Bremner, 1986b, or an alternative one). The relationship between the transformed slope (\( \alpha \)) and duration of the experiment (\( D \)), and between \( \alpha \) and temperature of the experiment (\( T \)), was assumed to be linear for experimental type (\( ET \)), sample pre-treatment (\( P \)), and extraction condition (\( EC \)). The model used to describe the data was, therefore:

\[
\alpha_{ijkm} = ET_i + P_k + EC_m + b \times D_{ijkm} + c \times T_{ijkm} + e_{ijkm}
\]

where \( \alpha_{ijkm} \) is the transformed slope (mg N per mg EON) for individual \( j \) in experimental type \( i \), sample pre-treatment \( k \) and extraction conditions \( m \); \( ET_i \) is the fixed effect of experimental type (\( i = 1, 2, 3, 4 \)), \( P_k \) and \( EC_m \) are the fixed effects of sample pre-treatment (\( k = 1, 2 \)) and extraction conditions (\( m = 1, 2 \)); \( D_{ijkm} \) is the duration of the experiment for individual \( j \) in experimental type \( i \), with sample pre-treatment \( k \), and extraction condition \( m \); \( T_{ijkm} \) is the temperature of the experiment for individual \( j \); \( b \) and \( c \) are the pooled within-group regression
coefficients to be estimated; and $e_{ijkm}$ are the random errors, assumed to be independent and $N(0, \sigma^2_e)$.

In addition, we analysed the variation among slopes in experiments with similar methodology. If these regression lines are strongly different from each other, then the applicability of regression lines will be small. We collected information on levels of EON and mineralizable N from short-term experiments (duration < 30 days) that were performed under controlled optimum conditions, either anaerobic ($n = 15$) or aerobic ($n = 19$). Studies using other extraction procedures than those proposed by Gianello & Bremner (1986b) were excluded. Short-term experiments were selected because the assumption of zero order kinetics is only valid for the initial phase of mineralization (Benbi & Richter, 2002). Linear regression coefficients were estimated with EON (mg kg$^{-1}$) as predictor of net N mineralization rate $k$ (mg kg$^{-1}$ day$^{-1}$) within each study. Levene's test was used to test whether the error variances of regression lines were significantly different among studies. Data from all studies were also pooled for both anaerobic and aerobic groups separately. The full model for each of these two groups is referred to as ‘full model 1’. A full model was also regressed after log transformation of $k$ and EON (when original data were not normally distributed) and removal of outliers (data points with a standardized residual bigger than three or a Cook’s Distance larger than one were removed); this model is referred to as ‘full model 2’.

3.3 Results and discussion

3.3.1 Relationship between EON and mineralizable N

For all soil tests, EON was positively related ($P < 0.05$) to (potentially) mineralizable N (Fig. 3.1). The strength of this relationship may be overestimated for some soil tests because our data were biased for soil tests measuring total hydrolysable N in 6 M HCl ($P < 0.001$), extractable organic N in hot CaCl$_2$ ($P < 0.007$), and extractable organic N in NaHCO$_3$ extracts ($P < 0.001$). Hence, experiments with better correlations between these EON fractions and N mineralization seemed to be more likely to be published than studies with poorer correlations.
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For all three soil tests, the fail-safe number according to Rosenthal’s (1979) method was larger than 5×n + 10: all EON fractions were positively related to (potentially) mineralizable N. The mean correlation coefficient r across all soil tests was 0.69, indicating that EON fractions generally explained 47% of the variation in (potentially) mineralizable N (n = 1675, P < 0.05). Total N explained 43% of the variation (n = 393, P < 0.05). These percentages are too small to allow accurate fertilizer recommendations according to the guidelines given by Malley et al. (2004), who suggested that R² values of calibrated soil tests should be more than 83%. This emphasizes the fact that current ‘single soil test’ approaches, consisting of the evaluation of simple linear relationships between EON and (potentially) mineralizable N, have to be improved in order to find a useful predictor of the latter. It also demonstrates that we need to understand why both EON and mineralizable N are related and which factors can be used to improve the relationship. One

Figure 3.1. Average variance (%) in N mineralization explained by EON for 20 soil tests compared with total N. Error bars are 95% confidence intervals. Grey bars are significantly different from total N (P < 0.05); white bars are not different from total N. Soil tests abbreviations are as in Table 3.1, and n is the number of observations.

For all three soil tests, the fail-safe number according to Rosenthal’s (1979) method was larger than 5×n + 10: all EON fractions were positively related to (potentially) mineralizable N.

The mean correlation coefficient r across all soil tests was 0.69, indicating that EON fractions generally explained 47% of the variation in (potentially) mineralizable N (n = 1675, P < 0.05). Total N explained 43% of the variation (n = 393, P < 0.05). These percentages are too small to allow accurate fertilizer recommendations according to the guidelines given by Malley et al. (2004), who suggested that R² values of calibrated soil tests should be more than 83%. This emphasizes the fact that current ‘single soil test’ approaches, consisting of the evaluation of simple linear relationships between EON and (potentially) mineralizable N, have to be improved in order to find a useful predictor of the latter. It also demonstrates that we need to understand why both EON and mineralizable N are related and which factors can be used to improve the relationship. One
important factor is the extraction procedure itself: the mean percentage explained variance in (potentially) mineralizable N by EON varied from 20 to 74% for different EON fractions (Fig. 3.1). Another factor is the accuracy of the biological method that is used as a reference.

Though biological methods have been used to calibrate chemical soil tests for decades, there is no consensus on which of them has to be used for a critical assessment of soil tests (Wang et al., 2001). Our meta-analysis suggests that biological methods estimating mineralizable N by net increases in inorganic N are better than N uptake or economic optimum N rates (data not shown). In addition, estimation of gross N mineralization may be preferred to net N mineralization because the net N rate represents a balance between mineralization and immobilization and both processes are regulated by different mechanisms (Wang et al., 2001). Unfortunately, our database contains almost no studies determining gross N mineralization rates and was therefore too limited to test this suggestion.

The strength of the relationship between EON and (potentially) mineralizable N may improve when more than one EON or labile C fraction, or other soil characteristics such as texture, are introduced into the regression model. For example, both EON and mineralizable N vary among texture classes (Hassink, 1992; Ros et al., 2009), and their relationship may be different for the different classes (Groot & Houba, 1995). In addition, Schomberg et al. (2009) showed that a combination of total N, EON and CO₂ production strongly improved the prediction of mineralizable N. Others have noted improvements after introduction of management history (Osterhaus et al., 2008) or soil drainage (Fox & Piekielek, 1984; Williams et al., 2007). Hence, changing the ‘single soil test’ approach to a ‘multiple component’ approach may improve the accuracy of predictions of (potentially) mineralizable N. Potentially relevant co-variables for the latter approach have yet to be identified and evaluated.

It is still unknown why EON and (potentially) mineralizable N are correlated. A causal relationship between them can only exist when the size of EON reflects the flow of N through it (Haynes, 2005). Subsequently, soils with greater
contents of EON have larger mineralization rates. Results from Appel & Mengel (1993), however, suggested that this hypothesis was incorrect; they found no relationship between initial size of EON and the flow of N through EON, using the mineralization rate of added crop residues as an estimate of the latter. A statistically significant relationship between the size of EON and (potentially) mineralizable N (Fig. 3.1) therefore suggests that they are simultaneously affected by another variable. Both EON and mineralizable N could be affected by qualitative aspects of organic matter such as the degree of humification or stabilization (Six et al., 2002). Northup et al. (1995), for example, reported that the concentration of polyphenol in pine litter controlled the release of DON and inorganic N. Others have suggested that EON, mineralizable N and biomass N are linked (Cookson & Murphy, 2004). More research is necessary to reveal the mechanisms behind the relationship between EON and (potentially) mineralizable N. Isotopic tracing of the $^{15}$N applied (Ros et al., 2010b) may be useful to calculate the fluxes through the different EON fractions and to distinguish between biologically non-active and active fractions.

3.3.2 Comparison of soil tests with total N

The majority of the soil tests performed either worse than or similarly to total N as a predictor of N mineralization (Fig. 3.1). Soil tests using phosphate buffer solutions, cold CaCl$_2$, Ba(OH)$_2$, cold KCl, HCl-hydrolysable NH$_4$ and HCl-hydrolysable total N performed in a similar way to total N and explained 33–61% of the variation in N mineralization (Fig. 3.1). HCl-extractable amino acids and amino sugars, H$_2$SO$_4$, alkaline KMnO$_4$, NaHCO$_3$, NaOH and EUF explained less variation in N mineralization than total N. This suggests that extracted organic N compounds do not represent an N fraction that is more actively involved in mineralization than total N. Consequently, EON fractions are not preferentially mineralized by micro-organisms (Appel & Mengel, 1993), nor are they more involved in mineralization as intermediates (Cookson et al., 2007) or as a proxy for microbial activity than total N (Jenkinson, 1968).

Extracts with acid K$_2$Cr$_2$O$_7$, acid KMnO$_4$, hot CaCl$_2$, hot water and hot KCl explained variation in N mineralization better than total N ($P < 0.05$). Or-
ganic N extracted with these methods explained 57–74% of the variation in N mineralization. Similar results were found when mean $R^2$ values were calculated for field, greenhouse and laboratory experiments separately (data not shown).

### 3.3.3 Influence of extraction intensity on the predictive value of EON

We found no inverse relationship between the intensity of extraction (expressed as % of total N extracted) and the performance of a soil test. Extraction intensity strongly varied among soil tests, where the proportion of EON to total soil N ranged from less than 5% by mild extractants to more than 35% by intensive extraction methods such as acid hydrolysis (Fig. 3.2). Mean EON levels varied between 0.1 and 963 mg kg$^{-1}$ (Fig. 3.2). When calculated for field, greenhouse and laboratory experiments separately, we still found no evidence for our third hypothesis, as shown for both incubation and field experiments (Fig. 3.3). This lack of relationship between extraction intensity and predictive value was also

![Figure 3.2](Image)

**Figure 3.2.** Mean EON content (left, % of total N; right, mg kg$^{-1}$ soil) for 20 soil tests. Error bars indicate SD, and $n$ is number of observations. Soil test abbreviations are as in Table 3.1. Note discontinuity in x-axis.
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shown by the strong variation among the six best soil tests (Fig. 3.1). Hence, although mild extractants selectively extract more biomass N from soil than intensive extractants (Kelley & Stevenson, 1985), this had no consistent impact on the predictive value of EON fractions. This suggests that biomass N also fails as an indicator of (potentially) mineralizable N. Similarly to EON, the turnover rate of microbial biomass may be more important than its size. This suggestion is supported by results of Holmes & Zak (1994), who showed that neither microbial biomass nor the net change in microbial biomass were correlated with daily rates of net N mineralization.

Alkali extractions resulted in smaller $r$ values than neutral or acidic salt solutions (Fig. 3.1). EON extracted with alkali may have contained relatively more inactive N: this is supported by the observation that organic compounds extracted with alkali but not with acid had a faster turnover time than the acid-extractable fraction (Trumbore, 2000).

![Graph showing relationship between extraction intensity and explained variance in mineralizable N](image)

**Figure 3.3.** Relationship between extraction intensity (expressed in % of total N) and mean % explained variance in mineralizable N by EON for 20 soil tests. Data are plotted for both field (grey) and incubation (black) experiments. Error bars on the x-axis are SD, and on the y-axis are 95% confidence intervals.
3.3.4 Differences between the laboratory and field

The strength of the relationship between EON and (potentially) mineralizable N was weaker ($P < 0.001$) when N mineralization was measured in field experiments ($R^2 = 17\%$, $n = 291$) compared with greenhouse experiments ($R^2 = 44\%$, $n = 567$), aerobic incubation ($R^2 = 54\%$, $n = 494$) and anaerobic incubation experiments ($R^2 = 64\%$, $n = 323$), when analysed for all soil tests. Similar differences were found when soil tests were analysed individually (data not shown). Percentage explained variance in N mineralization determined in laboratory or greenhouse experiments was, on average, $35 \pm 16\%$ ($\pm 1$ SD) greater than in experiments performed in the field (range, $8 - 72\%$), except for some organic fractions extracted with 6 M HCl. The improved performance of these HCl-extractable fractions in the field may have been related to the limited number of field data points available for this comparison ($n < 6$).

The difference in predictive value of EON between laboratory and field studies can be explained by greater variability in N mineralization measured in the field because of the range of effects of temperature, moisture, biomass, soil structure and losses by diffusion, denitrification, leaching or root uptake (Hatch et al., 2000). Although pot experiments in greenhouses have similar sources of variation to those in the field, the absolute variance is often smaller; spatial and temporal variability is reduced through pre-treatment of soil samples (sieving, drying and homogenization) and controlled temperature and moisture. Therefore, the predictive value of EON did not differ between pot and incubation experiments for most soil tests (data not shown).

Most of the soil tests (approximately 90\%) had mean $R^2$ values of less than 49\% for predicting mineralization in the field (data not shown). These values suggest that EON cannot be used as a reliable predictor in the field (Malley et al., 2004). In contrast, $R^2$ values for the six best soil tests under optimum laboratory conditions varied between 60 and 80\% (data not shown). This suggests that EON, particularly when used in a ‘multiple-component’ approach, may have some potential for accurate predictions under laboratory conditions. Correction
for differences between potential and actual mineralization may be done afterwards using simulation models (Campbell et al., 1997).

3.3.5 Variation in slopes among studies

Strong variation in calibrated slopes, between EON and net N mineralization rate, was observed when we compared regression lines for each chemical method (Fig. 3.4). For example, a 1-mg increase in hot CaCl₂-EON could correspond to a predicted mineralization rate varying between −0.01 and 0.28 mg N day⁻¹. When two soils with a slight difference in EON of 10 mg kg⁻¹ were incubated for 1 week, the use of regression lines for both minimum and maximum observed slopes resulted in a difference in predicted N mineralization of 21 mg kg⁻¹. Simi-

![Figure 3.3.](image)

**Figure 3.3.** Estimated mean slope of regression lines between EON and mineralizable N for 18 soil tests. Error bars represent the range between minimum and maximum slopes reported. Soil test abbreviations as in table 3.1, and n is number of observations. Soil tests are ranked from large to small EON contents. Note discontinuity in x-axis.
lar variation was observed for all EON fractions (Fig. 3.4). Hence, estimates using relationships from the literature can result in an enormous error in the prediction.

3.3.6 Factors affecting the strength of the relationship between hot KCl-EON and mineralizable N

Hot-KCl-extractable organic N was related to N mineralization in almost all experiments ($P < 0.05$), regardless of whether N mineralization was measured under laboratory or field conditions (Figs. 3.1 and 3.5). Although EON explained 60% of the variation in mineralizable N in laboratory experiments, this method is not accurate enough to improve fertilizer recommendations (Malley et al., 2004). The predicted value of EON was smaller when mineralization was measured under field than under laboratory conditions ($R^2_{\text{field}} = 37\%$; $P_{\text{diff}} < 0.001$).

![Figure 3.5](image-url)

**Figure 3.5.** Relationship between hot-KCl-extractable organic N (mg kg$^{-1}$) and net N mineralization rate (mg kg$^{-1}$ day$^{-1}$) for observations in aerobic and anaerobic incubation experiments. Data collected from 27 papers.
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indicating that this test also predicts the potential rather than the actual N mineralization. The correlation between EON and mineralizable N in field experiments is not only weaker because of greater variation in environmental conditions, but also because of greater uncertainty in the mineralization measurement itself. Use of derivative methods such as yield or N uptake, is probably less accurate than methods that account for possible losses and external N inputs. Indeed, R² values tended to increase from 34% for yield (n = 17) up to 45% for N uptake (n = 32), and up to 54% for net increases in inorganic N (n = 16).

Hot KCl-EON also performed better as a means of prediction in pot than in field experiments (P = 0.002). Use of undisturbed field-moist soil instead of dried, homogenized and sieved soil reduced the predictive value of EON as estimator of mineralizable N (P = 0.04); the averaged R² value was 51% for experiments using field-moist soils (n = 31) and 62% for experiments using homogenized and dried soils (n = 141). Application of fertilizer N resulted in a similar decrease; the averaged R² value of non-fertilized soils (n = 47) was at least 22% greater than R² value of the fertilized soils (n = 12; P = 0.003). This can be explained by increasing uncertainty in mineralizable N measurements. On the basis of these observations, we propose that the relationship between EON and mineralizable N should be calibrated under optimum conditions; all activities increasing the variation in EON or mineralizable N should be avoided.

Variation in extraction conditions among hot-KCl procedures had no influence on the correlation between EON and mineralizable N. When the predictive value of soil tests using the standard procedure of Gianello & Bremner (1986b) was compared with the predictive value of soil tests using other procedures (variation is shown in Table 3.1), then the difference in R² values between both was not significant for field experiments (P = 0.053), aerobic incubations (P = 0.7), anaerobic incubations (P = 0.3) or pot experiments (P = 0.6). More in-depth comparisons support this conclusion: the influence of soil solution ratios on the strength of the relationship between EON and mineralizable N was not significant (P = 0.1) when soils were extracted at 100°C for 240 minutes. Similar results were found for the influence of extraction temperature (P = 0.8) and duration (P = 0.6). Although the amount of extracted organic N increases with in-
creasing duration, temperature and soil solution ratios (Ros et al., 2009), this obviously does not affect its predictive value. Nevertheless, a change in EON will alter the regression line of its relationship with (potentially) mineralizable N. Therefore, there is a strong need for consistency when the extraction procedure has been developed for the above-mentioned relationship.

3.3.7 Factors affecting the slope of the relationship between hot-KCl EON and mineralizable N

Slopes of regression lines, between hot-KCl EON and mineralizable N, were not influenced by experimental type, or by temperature or duration of the experiment, because of large variations within treatment groups. Similarly, extraction temperature, sample pre-drying and soil solution ratios did not explain the variation present in regression slopes (data not shown). These observations for hot KCl indicate that there is another unknown variable responsible for the strong variation in regression slopes for most soil tests (Fig. 3.4). They also indicate that regression lines from one experiment cannot be applied to another; their applicability is limited to the situation under which the calibration experiment was performed. This conclusion is also valid for experiments with similar methodology, as shown for the slopes in 19 aerobic and 15 anaerobic incubation experiments (Tables 3.2 and 3.3). For example, the predicted N mineralization in a soil with a mean EON content of 28 mg kg\(^{-1}\) soil can range between 20 and 243 mg kg\(^{-1}\) for an aerobic incubation period of 14 days. Levene’s test showed that the error variances of the individual regression lines were significantly different among aerobic (model 2; F(18, 407) = 3.69, \(P < 0.001\)) and anaerobic incubations (model 2; F(14, 244) = 2.78, \(P = 0.001\)). Use of these full models, being generally applicable, is therefore not reasonable and both failed to predict the mineralization rate accurately: the standard error of the predicted rate was 1.71 mg kg\(^{-1}\) day\(^{-1}\) for aerobic and 3.62 mg kg\(^{-1}\) day\(^{-1}\) for anaerobic incubations (Tables 3.2 and 3.3). Nevertheless, the presence of large \(R^2\) values in some individual studies suggests that when a soil test is calibrated on a set of soils covering the total variation in soil characteristics within a local region, then the outcome may be accurate enough to improve fertilizer recommendations there. To be conclusive
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Table 3.2. Regression models for net N mineralization rate (k) in units of mg kg⁻¹ day⁻¹ on hot KCl-EON (EON in mg kg⁻¹) measured in short-term (< 30 days) aerobic incubations.

<table>
<thead>
<tr>
<th>Experimental details</th>
<th>n</th>
<th>R²adj</th>
<th>Regression line</th>
<th>SEP</th>
<th>Reference/ remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full model 1</td>
<td>457</td>
<td>0.42</td>
<td>k = 0.22 x EON − 0.1</td>
<td>2.4</td>
<td>all data; no transformation</td>
</tr>
<tr>
<td>Full model 2</td>
<td>426</td>
<td>0.54</td>
<td>k = 0.10 x EON + 0.8</td>
<td>1.7</td>
<td>outliers removed; log-transformed</td>
</tr>
<tr>
<td>40°C for 7 days</td>
<td>5</td>
<td>0.27</td>
<td>k = 0.62 x EON − 3.6</td>
<td>1.7</td>
<td>Hossain et al. (1996)</td>
</tr>
<tr>
<td>25°C for 7 days</td>
<td>17</td>
<td>0.39</td>
<td>k = 0.26 x EON + 0.6</td>
<td>2.0</td>
<td>Stockdale &amp; Roca (1994)</td>
</tr>
<tr>
<td>30°C for 7 days</td>
<td>14</td>
<td>0.13</td>
<td>k = 0.16 x EON + 0.6</td>
<td>2.6</td>
<td>Khoi et al. (2006)</td>
</tr>
<tr>
<td>30°C for 14 days</td>
<td>31</td>
<td>0.47</td>
<td>k = 0.19 x EON − 1.5</td>
<td>0.6</td>
<td>El-Karim &amp; Usta (2001)</td>
</tr>
<tr>
<td>35°C for 14 days</td>
<td>42</td>
<td>0.19</td>
<td>k = 0.11 x EON + 0.7</td>
<td>1.1</td>
<td>Julii el al. (1996)</td>
</tr>
<tr>
<td>30°C for 14 days</td>
<td>12</td>
<td>0.54</td>
<td>k = 0.23 x EON + 1.8</td>
<td>3.7</td>
<td>Wang et al. (2001)</td>
</tr>
<tr>
<td>30°C for 14 days</td>
<td>30</td>
<td>0.77</td>
<td>k = 0.21 x EON + 1.6</td>
<td>1.2</td>
<td>Gianello &amp; Bremner (1986a)</td>
</tr>
<tr>
<td>35°C for 14 days</td>
<td>29</td>
<td>0.65</td>
<td>k = 0.13 x EON − 0.1</td>
<td>1.0</td>
<td>Gianello &amp; Bremner (1986a)</td>
</tr>
<tr>
<td>30°C for 14 days</td>
<td>10</td>
<td>0.74</td>
<td>k = 0.33 x EON − 2.1</td>
<td>0.4</td>
<td>Hossain et al. (1996)</td>
</tr>
<tr>
<td>40°C for 14 days</td>
<td>5</td>
<td>0.30</td>
<td>k = 0.41 x EON − 2.4</td>
<td>1.1</td>
<td>Hossain et al. (1996)</td>
</tr>
<tr>
<td>30°C for 14 days</td>
<td>33</td>
<td>0.84</td>
<td>k = 0.25 x EON − 1.1</td>
<td>1.2</td>
<td>Gianello &amp; Bremner (1986b)</td>
</tr>
<tr>
<td>35°C for 14 days</td>
<td>33</td>
<td>0.86</td>
<td>k = 0.23 x EON − 0.2</td>
<td>0.2</td>
<td>Sharif et al. (2003)</td>
</tr>
<tr>
<td>25°C for 14 days</td>
<td>8</td>
<td>0.90</td>
<td>k = 0.13 x EON + 0.6</td>
<td>1.1</td>
<td>Curtis &amp; Woa (1999)</td>
</tr>
<tr>
<td>30°C for 14 days</td>
<td>14</td>
<td>0.38</td>
<td>k = 0.12 x EON + 4.6</td>
<td>1.1</td>
<td>Khoi et al. (2006)</td>
</tr>
<tr>
<td>25°C for 24 days</td>
<td>60</td>
<td>0.62</td>
<td>k = 0.16 x EON + 0.1</td>
<td>0.3</td>
<td>Picou et al. (2002)</td>
</tr>
<tr>
<td>25°C for 24 days</td>
<td>16</td>
<td>0.67</td>
<td>k = 0.23 x EON + 1.6</td>
<td>0.4</td>
<td>Soon et al. (2007)</td>
</tr>
<tr>
<td>40°C for 28 days</td>
<td>5</td>
<td>0.36</td>
<td>k = 0.28 x EON − 1.5</td>
<td>0.7</td>
<td>Hossain et al. (1996)</td>
</tr>
<tr>
<td>30°C for 28 days</td>
<td>14</td>
<td>0.17</td>
<td>k = 0.05 x EON + 2.9</td>
<td>0.8</td>
<td>Khoi et al. (2006)</td>
</tr>
</tbody>
</table>

n = number of soils included in the regression; R²adj = percentage explained variance; SEP = standard error of the prediction (mg kg⁻¹ day⁻¹). Experimental details refer to the measurement of the mineralization rate.

Table 3.3. Regression models for net N mineralization rate (k) in units of mg kg⁻¹ day⁻¹ on hot KCl-EON (EON in mg kg⁻¹) measured in short-term (< 30 days) anaerobic incubations.

<table>
<thead>
<tr>
<th>Experimental details</th>
<th>n</th>
<th>R²adj</th>
<th>Regression line</th>
<th>SEP</th>
<th>Reference/ remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full model 1</td>
<td>263</td>
<td>0.15</td>
<td>k = 0.13 x EON + 4.0</td>
<td>4.1</td>
<td>all data</td>
</tr>
<tr>
<td>Full model 2</td>
<td>259</td>
<td>0.30</td>
<td>k = 0.23 x EON − 2.4</td>
<td>3.6</td>
<td>outliers removed</td>
</tr>
<tr>
<td>40°C for 7 days</td>
<td>31</td>
<td>0.35</td>
<td>k = 0.61 x EON − 0.7</td>
<td>2.6</td>
<td>El-Karim &amp; Usta (2001)</td>
</tr>
<tr>
<td>30°C for 14 days</td>
<td>19</td>
<td>0.38</td>
<td>k = 0.20 x EON + 3.6</td>
<td>3.8</td>
<td>Wang et al. (2001)</td>
</tr>
<tr>
<td>40°C for 7 days</td>
<td>12</td>
<td>0.09</td>
<td>k = 0.05 x EON + 12</td>
<td>3.4</td>
<td>Wang et al. (1996)</td>
</tr>
<tr>
<td>30°C for 7 days</td>
<td>30</td>
<td>0.89</td>
<td>k = 0.47 x EON + 0.2</td>
<td>1.7</td>
<td>Gianello &amp; Bremner (1986a)</td>
</tr>
<tr>
<td>20°C for 21 days</td>
<td>15</td>
<td>0.80</td>
<td>k = 0.02 x EON + 0.5</td>
<td>0.4</td>
<td>Velthof et al. (2000)</td>
</tr>
<tr>
<td>40°C for 7 days</td>
<td>15</td>
<td>0.63</td>
<td>k = 0.97 x EON − 4.7</td>
<td>1.6</td>
<td>Hossain et al. (1996)</td>
</tr>
<tr>
<td>40°C for 7 days</td>
<td>12</td>
<td>0.71</td>
<td>k = 0.49 x EON + 0.7</td>
<td>0.4</td>
<td>Illa'sva &amp; Waring (1992)</td>
</tr>
<tr>
<td>40°C for 7 days</td>
<td>12</td>
<td>0.21</td>
<td>k = 0.06 x EON + 1.4</td>
<td>1.4</td>
<td>Scott et al. (2005)</td>
</tr>
<tr>
<td>40°C for 7 days</td>
<td>16</td>
<td>0.83</td>
<td>k = 0.96 x EON − 0.3</td>
<td>1.1</td>
<td>Soon et al. (2007)</td>
</tr>
<tr>
<td>40°C for 7 days</td>
<td>17</td>
<td>0.38</td>
<td>k = 0.18 x EON − 0.1</td>
<td>1.4</td>
<td>Stockdale &amp; Roca (1994)</td>
</tr>
<tr>
<td>40°C for 7 days</td>
<td>8</td>
<td>0.63</td>
<td>k = 0.50 x EON + 0.7</td>
<td>0.4</td>
<td>Waring et al. (1994)</td>
</tr>
<tr>
<td>40°C for 7 days</td>
<td>33</td>
<td>0.90</td>
<td>k = 0.38 x EON + 1.4</td>
<td>1.4</td>
<td>Gianello &amp; Bremner (1986b)</td>
</tr>
<tr>
<td>20°C for 7 days</td>
<td>15</td>
<td>0.56</td>
<td>k = 0.47 x EON − 0.1</td>
<td>1.5</td>
<td>Beauchamp et al. (2003)</td>
</tr>
<tr>
<td>40°C for 7 days</td>
<td>5</td>
<td>0.27</td>
<td>k = 0.05 x EON + 1.5</td>
<td>0.7</td>
<td>Moroni et al. (2004)</td>
</tr>
<tr>
<td>5°C for 7 days</td>
<td>18</td>
<td>0.84</td>
<td>k = 0.17 x EON + 4.1</td>
<td>1.8</td>
<td>Khoi et al. (2006)</td>
</tr>
</tbody>
</table>

n = number of soils included in the regression; R²adj = percentage explained variance; SEP = standard error of the prediction (mg kg⁻¹ day⁻¹). Experimental details refer to the measurement of the mineralization rate.
on this point, the calibrated regression equations need to be validated in independent field experiments.

3.4. Conclusions

Although soil tests extracting organic N were positively related (P < 0.05) to (potentially) mineralizable N, they were not accurate enough for introduction into fertilizer recommendation schemes. The $R^2$ values of their relationship with mineralizable N ranged between 20 and 74%. On the basis of this analysis, using results from the last 100 years, we may conclude that the ‘single soil test’ approach, calibration of a relationship between a single EON fraction and (potentially) mineralizable N, is not likely to result in an accurate prediction of (potentially) mineralizable N.

We recommend a change to a ‘multiple-component’ regression approach, in which the prediction of (potentially) mineralizable N is not based on one single soil test, but on a combination of soil tests and soil properties. The additional components may include different soil tests extracting organic N or C, but also site-specific information such as texture, groundwater level and land use. Potentially relevant variables have to be identified and evaluated using multiple regression techniques. This ‘multiple components’ approach will predict the mineralization more accurately, in particular when it is determined under optimum and constant environmental conditions. Additional correction for soil moisture and temperature remains necessary to obtain reliable predictions in the field, and hence the ‘multiple components’ approach may also need to include simulation modelling to correct for these environmental factors.

The factors that affect the slope of the regression of EON and (potentially) mineralizable N should be further investigated to determine whether these depend on soil properties such as texture, organic matter, acidity and groundwater level, or methodological issues such as location, climate and time of sampling. Applicability of soil tests remains limited to the conditions of the calibration set unless the variables that cause the strong variation in regression lines are identified.
Most importantly, however, our results emphasize the limited knowledge of the functional role of EON in N mineralization, and its interaction with soil properties and environmental conditions. Further research is needed to understand why EON and mineralizable N are related, and how a mechanistically meaningful organic N fraction can be extracted. Isotopic tracing of $^{15}$N may be useful in calculating the fluxes through the different fractions and in distinguishing between biologically non-active and active fractions. Our results suggest that neutral or acidic salts extract a more relevant N fraction, regardless of their extraction intensity, but more research is necessary to confirm this suggestion.

Acknowledgement

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PREDICTING SOIL N MINERALIZATION

RELEVANCE OF ORGANIC MATTER FRACTIONS AND SOIL PROPERTIES

Gerard H. Ros
Marjoleine C. Hanegraaf
Ellis Hoffland
Willem H. van Riemsdijk
Abstract

Distinct extractable organic matter (EOM) fractions have been used to assess the capacity of soils to supply nitrogen (N). However, substantial uncertainty exists on their role in the N cycle and their functional dependency on soil properties. We therefore examined the variation in mineralizable N and its relationship with EOM fractions, soil physical and chemical properties across 98 agricultural soils with contrasting inherent properties and management histories. Mineralizable N was determined by aerobic incubation at 20°C and optimum moisture content for 20 weeks. We used multivariate statistical modelling to account for multi-collinearity, an issue generally overlooked in studies evaluating the predictive value of EOM fractions. Mineralization of N was primarily related to the size of OM pools and fractions present; they explained 78% of the variation in mineralizable N whereas other soil variables could explain maximally 8%. Both total and extractable OM expressed the same soil characteristic from a mineralization perspective; they were positively related to mineralizable N and explained a similar percentage of the variation in mineralizable N. Inclusion of mineralizable N in fertilizer recommendation systems should be based on at least one OM variable. The most appropriate EOM fraction can only be identified when the underlying mechanisms are known; regression techniques are not suitable for this purpose. Combination of single EOM fractions is not likely to improve the prediction of mineralizable N due to high multi-collinearity. Inclusion of texture related soil variables or variables reflecting soil organic matter quality may be neglected due to their limited power to improve the prediction of mineralizable N.

4.1 Introduction

Soil organic matter (SOM) strongly affects soil fertility, biomass production, species composition and carbon (C) sequestration in terrestrial ecosystems (Reich et al., 1997; Tiessen et al., 2002; Haynes, 2005). The content of SOM, and its quality, is therefore regarded as a key factor for the capacity of soils to sustain biological productivity, to maintain environmental quality, and to promote plant and animal health. Distinct SOM fractions extractable with chemical salt solutions have been used to assess ecosystem services of SOM, in particular for its ability to supply N. However, none of these SOM fractions has been universally accepted as an indicator of the soils’ capacity to supply N and substantial uncertainty exists on their role in the N cycle. Hence, a better understanding of their function and interrelationships with other soil properties is important to improve our ability to predict how terrestrial ecosystems will respond to soil and nutrient management, and to global climate change. In particular, an accurate estimate of the N mineralized from SOM will improve the sustainability of agriculture because it allows farmers to determine the rate of N fertilizer application required to optimize crop yield and to minimize N losses to the environment.

Soil organic matter is a heterogeneous mixture of organic compounds varying in age, molecular structure, stability, nutrient content, and biological availability. Consequently, the functional importance of SOM varies systematically, with the youngest compounds being most biologically active, and materials of recent origin and intermediate age contributing notably to the physical status of soils (Wander, 2004). Compounds with longer residence times exert more influence on the physicochemical reactivity of soils. Realizing that the total SOM pool includes this continuum of compounds, numerous attempts have been made to identify and to characterize functionally distinct organic matter (OM) pools and fractions (Zsolnay, 1996; Wander, 2004; Haynes, 2005), in particular with respect to N dynamics in soil (Ros et al., 2011b).

In this context, the term pool refers to theoretically separated, kinetically delineated components of SOM, whereas the term fraction is used for measura-
ble OM components. Soil organic matter models distinguish various OM pools and are used to characterize their size, turnover rate and degree of stabilization based on time series of decomposition data (e.g., Smith et al., 1997). In contrast, chemical fractionation methods are designed to isolate chemically labile from persistent OM, and the chemically labile fraction may represent the most biologically available fraction of total SOM. These methods extract a specific organic nitrogen or organic carbon (C) fraction based on its solubility in water with and without electrolytes or in organic solvents, on its hydrolysability with water or acids, and its resistance to oxidation. The extracted organic N fraction is often termed dissolved organic N, soluble organic N, or extractable organic N. We use the term extractable organic matter (EOM) when explicit details on the elemental composition of the extracted organic matter are not necessary. When referring to the specific elemental composition of EOM, we use the abbreviation EON for extractable organic N and EOC for extractable organic C.

The relevance of EOM fractions is usually inferred from its correlation with a biological ‘reference’ criterion that is supposed to reflect the N mineralization capacity of soils (Wang et al., 2001). When this ‘reference’ criterion has been estimated from long-term laboratory incubations (time scale 0.5 to 3 years), it is often called potentially mineralizable N, bioavailable N or N supplying capacity (Stanford & Smith, 1972; Griffin, 2008; Nannipieri & Eldor, 2009). This potentially mineralizable N pool is usually estimated along with its mineralization rate constant using a first-order exponential function. The fraction that mineralizes in short term laboratory incubations and field experiments (time scale usually between 1 and 8 weeks) is often called net N mineralization or (actual) mineralizable N (Ros et al., 2011b). The difference between both approaches is that the potentially mineralizable N pool reflects the amount of organic N that can be mineralized (no environmental constraints) whereas the actual mineralizable N pool is the fraction that actually mineralizes (depending on environmental conditions). Both the potential and actual mineralizable N have been used to assess the predictive value of EOM fractions. However, none of the EOM fractions is universally accepted as an estimate of mineralizable N.
Relevance of SOM fractions and soil properties

The lack of agreement can be attributed to, among others, inconsistencies with results from actual field requirements under differing climatic conditions where crop demand, as well as the soil N supplying capacity vary (Mosier et al., 2004). In addition, identification of appropriate fractionation methods is hampered by huge variation among data sets and calibration conditions (Ros et al., 2011b). Some therefore indicate that chemical fractionation methods only provide a relative indication of the (potentially) mineralizable N pool (Bundy & Meisinger, 1994; Stockdale et al., 1997). Others indicate that EOM fractions have to be used in conjunction with each other (Schomberg et al., 2009), with other soil properties (Gallagher & Bartholomew, 1964), or with simulation models (Stockdale et al., 1997; Campbell et al., 1997) to provide reliable estimates of (potentially) mineralizable N.

An extensive number of studies certainly have related (potentially) mineralizable N, or its turnover, to soil properties and environmental conditions (e.g., Connell et al., 1995; Strong et al., 1998; Van Eekeren et al., 2010). Soil OM fractions, such as the extractable OM fraction with hot water, hot KCl or 0.01M CaCl₂, are usually positively related with (potentially) mineralizable N (e.g. Sharifi et al., 2007; Schomberg et al., 2009), whereas an increase in C-to-N ratio is associated with a decrease in mineralization rates (e.g. Riffaldi et al., 1996). Mineralization of organic N is often negatively affected by clay content, likely due to OM binding to mineral particles and biomass preservation (Hassink, 1992; 1997). Low P availability is also known to restrict nitrification, but this limitation seems to be rare in fertilized agricultural soils (Tate & Salcedo, 1988; Carlyle et al., 1990). Soil pH, moisture, and temperature are often nonlinearly related with the dynamics of N (Rodrigo et al., 1997; Paul et al., 2003). Introduction of these soil properties in regression models is rare, although several studies have shown that the slope of the regression line between EOM fractions and (potentially) mineralizable N can be different among soils varying in texture, pH, land use, and drainage classes (Verstraeten et al., 1970; Stanford & Smith, 1978; Fox & Piekielek, 1984; Groot & Houba, 1995). Continued work is essential to accumulate critical experimental evidence across a wide range of soils to iden-
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tify appropriate EOM fractions, and their interaction with soil properties, helping us to understand the role of SOM in the N cycle.

Combining SOM fractions and soil properties into multiple regression models has been shown to improve the prediction of (potentially) mineralizable N (Schomberg et al., 2009; Dessureault-Rompré et al., 2010). However, the biological meaning of the components of these statistical models is often unclear, and strong correlations between the various factors may confound the underlying mechanisms. Neglecting the issue of collinearity hampers our mechanistic understanding and subsequently the identification of generally applicable EOM fractions and soil properties for improvement of fertilizer recommendations. For example, extractable OM fractions have been favoured above total OM as a predictor of (potentially) mineralizable N due to the release of labile OM compounds during extraction (Haynes, 2005), but there is no evidence that the turnover of labile OM (being a source of N) is reflected in the size of EOM fractions (Ros et al., 2010c). Understanding the underlying mechanisms may improve the applicability of EOM fractions in fertilizer recommendation systems across climate zones, land uses and soil types, since current relationships seem to be only valid for the set of soils from which they were derived (Ros et al., 2011b). Use of multivariate statistical modelling may be preferred above classical regression techniques since it takes the collinearity among soil variables into account and combines the variables in such a way that the underlying soil factors relevant for the prediction of (potentially) mineralizable N might be identified. As far as we know, multivariate statistical techniques have not been applied in this field.

We examined the variation in mineralizable N and its relationship with EOM fractions and other soil properties across 98 agricultural soils with contrasting inherent properties and management histories using multivariate statistical modelling. More specifically, we focus on the following research questions: (1) Does the addition of soil properties other than EOM fractions to statistical models improve the predictive power of these models to explain the variation in mineralizable N? (2) Are there significant differences among total and extractable OM fractions in their ability to explain the variation in mineralizable N? (3) Does the combination of EOM fractions result in serious improvement
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of models predicting mineralizable N? Answering these questions allows us to elaborate on how EOM fractions and other soil properties can be used in fertilizer recommendation systems to improve the sustainability of N management in agricultural ecosystems.

4.2 Material and methods

4.2.1 Soils
Soil samples were collected from 98 agricultural soils from various areas of the Netherlands. Soils were sampled in spring and early summer 2009. Each sample (2-4 kg) was a mixture of 100 subsamples taken in a 'W' pattern across the selected field. Samples were taken with an auger at 0-30cm depth (arable soils) or 0-10cm depth (grassland soils). A subsample was dried (40 °C) and passed through a 2mm sieve. Large root fragments found after sieving were discarded, along with all soil particles larger than 2 mm.

4.2.2 Incubation experiment
We used a biologically based, short-term aerobic incubation method to estimate the size of mineralizable N. We moistened 100g of field moist soil (sieved < 5mm) to 60% of the maximum water holding capacity, and incubated it for 140 days at 20 °C in gas permeable plastic bags (Audiothene 0.10 mm, Art. No. A15100). Duplicate bags were destructively sampled after 0, 14, 84, and 140 days, and the soil was analyzed for EOC, EON, NH₄ and NO₃ (Houba et al., 2000).

We define mineralizable N as the amount of N that mineralizes over 140 days under constant environmental conditions (temperature is 20 °C; moisture content at field capacity). Calculation of a potentially mineralizable N pool using first order kinetics (Stanford & Smith, 1972; Wang et al., 2003) was not possible since about 50% of the soils showed a linear increase in inorganic N over 140 days of incubation.

4.2.3 Chemical and physical analyses
The pH, SOM, clay (<2 um), and cation exchange capacity (CEC) were determined using standard analytical procedures (Houba et al., 2000); pH was meas-
ured in a settling 1:5 (wt/vol) suspension of soil in 0.01 M CaCl₂, SOM by loss-on-ignition (550 °C), clay by the sieve and pipette method (NEN 5753), and cation exchange capacity (CEC) by the unbuffered 0.01 M BaCl₂ method (Gillman, 1979). The water-holding capacity (WHC) was determined by the addition of demineralized water to the soil until it became saturated and excess water was draining freely. The mass of water added was recorded. **Total N** was measured after soil digestion with a mixture of H₂O₂ and H₂SO₄ under the influence of Se as a catalyst (Novozamsky *et al.*, 1983). Initial concentrations of NH₄⁺, NO₃⁻, and **total extractable N** (TEN) were determined spectrophotometrically using a Segmented Flow Analyzer (SFA, Skalar, The Netherlands; Houba *et al.*, 2000). Total extractable N was determined after digestion of organic compounds by potassium persulfate (Houba *et al.*, 2000). **Extractable organic N** (EON) was subsequently calculated as the difference between TEN and inorganic N for both field moist (moist EON) and dried (dried EON) extracted soils. **Total EOC** was also measured in the CaCl₂ extract of moist (moist EOC) and dried soil (dried EOC), and subsequently analyzed using an automatic carbon analyzer (Skalar, The Netherlands; Houba *et al.*, 2000). **Hot water extractable C** (HWC) was determined according a modified method of Ghani *et al.* (2003). In short, moist soil samples (~ 3 gram dried) were extracted with 30 ml of distilled water for 16 hours in a hot water bath at 80 °C. Extracts were analyzed for C using an automatic carbon analyzer, after centrifugation (20 min, 3500 rpm) and filtration (0.45 μm). The **P status** of the soil was characterized using the 0.01 M CaCl₂ (P-CaCl₂, Houba *et al.*, 2000) and the PAL method (Egner *et al.*, 1960). The value of PAL was also used to express the availability of P in relation to C and N, expressed as the C-to-P and N-to-P ratio.

### 4.2.4 Statistical analyses

All data corresponded to a normal distribution after natural log transformation. Pearson correlation tests were used to evaluate the relationships between individual variables. Partial least squares (PLS) regression on the transformed data was used to model the relationships between mineralizable N, and the chemical and physical soil characteristics. The PLS regression creates a model of latent
variables, components, in which the information from the soil physical and chemical characteristics is combined in such a way that it maximizes the explanation of variation in mineralizable N (Geladi & Kowalski, 1986; Esposito Vinzi et al., 2010). The PLS regression is used in a similar way to multiple regression analysis, but PLS can handle correlated variables, which often preclude the use of multiple regression (James & McCulloch, 1990).

The predictive power of the PLS models was tested with full cross validation where one sample was excluded from the model and predicted by the model using the rest of the samples. The predictive ability was expressed as the explained variance of the cross validation predictions. We used the predictive residual error sum of square (PRESS) as an index to select how many components we have to include in the regression model. Components that explain less than 1% of the variation in mineralizable N were additionally excluded. Prior to the PLS modeling, all variables were scaled to unit variance. Data were analyzed with Genstat 13th.

4.3 Results

4.3.1 Range of soil physical and chemical characteristics

The soils varied widely in physical and chemical characteristics, and also in cumulative N mineralization (Table 4.1). Total N varied from 0.66 to 7.04 g kg⁻¹, total C from 5.3 to 129 g kg⁻¹, soil pH from 4.4 to 7.5, and clay content from <1 to 65%. The initial concentration of inorganic N varied between 4 and 129 mg kg⁻¹, representing 0.2 to 5% of total N. Total EOC varied between 42 and 521 mg kg⁻¹ (0.1-1.8% of total C) in previously dried soils, whereas HWC varied between 0.24 and 4.68 g kg⁻¹ (1.4-8.7% of total C). Extraction of soils with hot water resulted in a 10-fold increase in the release of organic C compared to total EOC determined in a 0.01 M CaCl₂ extract. Drying of soils also increased levels of EOC and EON (Table 4.1). The wide range in soil properties provided a useful data set for examining relationships between mineralizable N, EOM fractions and the chemical and physical characteristics of the soils.
4.3.2 Size and dynamics of the biologically available N pool

Cumulative inorganic N increased on average during 20 weeks to 34 mg kg\(^{-1}\) in the loamy soils, 36 mg kg\(^{-1}\) in the clayey soils, 85 mg kg\(^{-1}\) in the sandy soils, and to 123 mg kg\(^{-1}\) in the former peat soils (Fig 4.1). Land use had also a strong effect on the cumulative N mineralized with higher values for grassland than for arable soils (data not shown), mainly due to higher total OM levels. When nor-
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4.3.3 Relationship of mineralizable N with soil properties

Mineralizable N was strongly related \((P < 0.001)\) with hot water C \((r = 0.88)\), moist EON \((r = 0.80)\), moist EOC \((r = 0.79)\), dried EON \((r = 0.77)\), NH\(_4\) \((r = 0.75)\), C-to-P ratio \((r = 0.74)\), total C \((r = 0.73)\), N-to-P ratio \((r = 0.69)\), dried EOC \((r = 0.69)\), total N \((r = 0.67)\), water holding capacity \((r = 0.65)\), and initial moisture content \((r = 0.61)\). Extractable fractions of OM tended to be more strongly related with mineralizable N than total OM pools, in particular for those extracted on field moist soil. Less important soil variables affecting mineralizable N were pH \((r = -0.46, P < 0.001)\), PAL \((r = -0.47, P < 0.001)\), EON moist normalized for total N \((r = 0.46, P < 0.001)\), HWC normalized for total C \((r = 0.44, P < 0.001)\), C-to-N ratio \((r = 0.36, P < 0.001)\), clay content \((r = -0.31, P < 0.002)\), and dried EOC nor-

Figure 4.1. Cumulative N mineralization for restored peat soils \((n = 10)\), clayey soils \((n = 15)\), and sandy soils \((n = 69)\) over 140 days of incubation. Error bars denote ±1 SE of the mean. Data from loamy and peat soils are not shown for visual clarity.
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Correlations among soil properties are given as supporting Information (Appendix A3, Table A3.1). Analysis of the collinearity statistics derived from multiple regression statistics showed that 56 to 99% of the variance in one of the EOM fractions or soil properties could be explained by the others. The minimum calculated variance inflation factor (VIF) of all variables was 2.3, indicating that there is high multi-collinearity among them (a VIF greater than 2 is usually problematic).

4.3.4 Predicting mineralizable N by EOM fractions and soil properties

Mineralizable N was more strongly correlated to variables reflecting organic matter content than to any other variable (Fig 4.2). The PLS model combined the EOM fractions and soil properties in three latent components, from which the most important one could be interpreted as an organic matter component (component 1, Fig 4.2.A). This organic matter component of the PLS model, explaining 79% of the variation in mineralizable N, could satisfactorily be explained by HWC (90%), moist EOC (83%), total C (77%), dried EON (72%), total N (71%), moist EON (71%), and dried EOC (67%). Their positive relationship among each other and with mineralizable N indicated that an increase in total OM and EOM is generally associated with an increase in mineralizable N, and subsequently in the net N mineralization rate during incubation. Extractable OM fractions in general did not explain more of the variation in mineralizable N than total organic C and N, although single fractions like HWC could explain 13% more of the variation in the OM component than total C (Fig 4.2).

In contrast, none of the EON fractions explained more of the variation in mineralizable N than total N; the differences among them were relatively small (< 2%). Variables reflecting the quality of the organic matter, (e.g., C-to-N ratio of SOM, and the extractable C and N fractions normalized for total C and N, respectively), soil texture and pH had a minor influence ($r^2 < 21\%$) on the first PLS component. In contrast, the C-to-P and N-to-P ratio could significantly explain more than 74% of the variation in the OM component. The concentration of
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Inorganic P (P-CaCl₂) or total available P (PAL) had only a minor influence on mineralizable N (r² in the OM component < 34%), indicating that the mineralization of N was not limited by the availability of P.

The second and third component explained only 4% of the variation in mineralizable N (Figs 4.2.B and 4.2.C). Straightforward interpretation of these components is therefore complicated. The second component likely reflected the influence of soil texture, since mineralizable N was negatively correlated with texture dependent variables such as clay content and CEC; the percentage explained variance in this component was 31% for clay content, and 64% for CEC (Fig 4.2.B). The same component was positively correlated with EOM fractions.
when normalized for total OM; the percentage explained variance was 66% for HWC, 25% for moist EOC, 12% for dried EOC, 56% for moist EON, and 49% for dried EON. The factors of the third component explained too little variance to interpret this component, but it probably depends on the initial concentration of NO$_3$ (Fig 4.2.C). This NO$_3$ effect may be due to an artefact of our dataset, since 15 out of the 98 soils had high initial NO$_3$ levels due to the fact that they were sampled in 2008 and stored for one year (at 1-2 °C), as opposed to the other soils that were sampled shortly before the incubation experiment started. This is confirmed by the fact that the number of significant components reduces from three to two when NO$_3$ is left out of the PLS analysis.

4.4 Discussion

4.4.1 Role of OM and soil properties in determining mineralizable N

Mineralization of N in agricultural soils is primarily related to the size of total and extractable organic matter fractions. In our research, the OM component explained 79% of the variation in mineralizable N whereas the other components only explained 8% (Fig 4.2). Hence, fertilizer recommendation systems using an estimate of mineralizable N should at least rely on a soil variable reflecting the size of the OM pool. The influence of variables reflecting soil texture or OM quality (e.g., C-to-N ratio and the extractable C and N fractions normalized for total C and N, respectively) was small compared to the influence of the size of the total OM pool, and they have consequently less potential to improve the accuracy of mineralizable N predictions. Similarly, Hofman et al. (2004) showed that microbial characteristics like basal and potential respiration corresponded positively with organic matter content of soils, while clay content, CEC, and pH played a minor role. The importance of SOM fractions for N mineralization is also corroborated by numerous studies evaluating the predictive value of single extractable OM fractions for the prediction of mineralizable N (reviewed in: Keeney, 1982; Griffin, 2008, Ros et al., 2011b). The observed dominant role of OM in determining N mineralization may explain and justify why much more effort has been taken to evaluate the predictive value of EOM fractions for the
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estimation of (potentially) mineralizable N compared to other soil properties as pH, water holding capacity, and clay content.

Introduction of texture-related soil variables besides an OM fraction may improve the prediction of mineralizable N, but this improvement is at maximum 4% as shown for the second model component (Fig 4.2.B). This component was dominated by clay content, CEC, pH, and qualitative characteristics of the organic matter involved. Clay strongly affects stabilization of organic N directly and through the formation of aggregate protected OM and preservation of microbial biomass (Hassink, 1992; 1997). Ammonium fixing clays can also stabilize N as NH$_4$ (Stevenson, 1986). We indeed found a negative influence of clay content on mineralizable N in both the first and second model component (Fig 4.2), in agreement with the lower cumulative N mineralization in clayey compared to sandy soils (Fig 4.1). This might suggest that this model component reflects a degree of SOM stabilization: soils with higher levels of clay have a lower mineralizable N value. Because clayey soils have a higher degree of stabilization, less OM can be extracted with weak extraction procedures (e.g. CaCl$_2$ or hot water). This explains why within the same model component mineralizable N was positively associated with moist EON, dried EON, HWC, and moist EOC when these fractions are normalized for total N or total C respectively (Fig 4.2.B): soils with higher levels of clay release relatively less OM during extraction compared to soils with lower levels of clay.

Surprisingly, the C-to-P and N-to-P ratio also explained a significant part of the variation in mineralizable N (Fig 4.2.A), indicating that the availability of P affects the net N mineralization in agricultural ecosystems. However, our soils seems not to be deficient in P since the concentration of P-CaCl$_2$ ranged between 0.3 and 17 mg kg$^{-1}$, being above the critical limits reported (Carlyle et al., 1990; Güsewell & Verhoeven, 2006). Consequently, the predictive value of C-to-P and N-to-P ratios for mineralizable N reflect their relationship with total OM; both ratio’s were indeed strongly correlated with total C and N ($P < 0.001$).
4.4.2 Total and extractable OM fractions

Our analysis showed that both total OM and extractable OM fractions are in the same model component, being positively related to N mineralization, and explaining a similar percentage of the variation in mineralizable N (Fig 4.2.A). This similarity among total and extractable OM fractions suggests that they all reflect the availability of the same OM pool and none of them is a priori preferable above the others as indicator of mineralizable N. Similarly, Ros et al. (2011b) concluded from their meta-analysis of the performance of soil N tests that all EOM fractions were positively correlated with indices of mineralizable N. In addition, they found that the predictive value of most common EOM fractions did not differ from total N. These findings contrast with the concept that chemically extracted OM fractions are a more sensitive indicator of the soils’ N mineralization potential than total N due to preferential release of biologically available OM (Haynes, 2005; Sharifi et al., 2007). Nevertheless, both total and extractable OM were strongly related to mineralizable N, indicating that they can be useful for improvement of the sustainability of N management in agricultural ecosystems. The best estimate was given by the amount of C released during hot water extraction (Fig 4.2.A); it explained 77% of the variation in mineralizable N when applied in a single relationship ($P < 0.001$; Fig 4.3). Similar significant relationships between HWC and mineralizable N have been found by others (Keeney & Bremner, 1966; Ghani et al., 2003; Van Eekeren et al., 2010).

Although all EOM fractions were present within one OM component, this does not necessarily imply that they are similarly involved in N mineralization. Generally, there are three mechanisms that can explain their positive relationship with mineralizable N (Ros et al., 2011b). First, EOM fractions can exist as a bio-available N pool in soils, being formed through depolymerization of SOM and functioning as a source of N; an increase in EOM indicates an increase in bio-available OM. Second, EOM fractions can reflect another soil variable responsible for N mineralization, such as biomass, which on its term depend on the size and quality of the OM present. Third, EOM fractions can be the leftover of decomposition; an increase in size indicates a higher turnover of bio-available OM. Strong differences in size and characteristics of our EOM fractions (see section
suggests that hot water EOM is an example of the first or second mechanism whereas the OM fraction extracted with CaCl₂ are examples of the second or third mechanism. However, the underlying mechanisms for these EOM fractions are still unknown, and further research is required to unravel their function in N mineralization processes.

4.4.3 Combining EOM fractions to estimate mineralizable N

Because no single EOM fraction has proven robust enough for broad acceptance, Schomberg et al. (2009) indicated that combining them improves the prediction of (potentially) mineralizable N. They found that a combination of total N and the CO₂ released from soil during a 3 day incubation was the best combination to estimate potentially mineralizable N, probably reflecting the size of the microbial biomass (CO₂ production) and the more recalcitrant fraction of organic N (total N). However, results of multiple regression models may not have a simple biological basis for interpretation, limiting the application range of calibrated statistical models. Our analysis questions whether a combination of EOM frac-

Figure 4.3. Relationship between hot water extractable C (HWC) and mineralizable N. Both axis were ln-transformed
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...lations will result in serious improvement of the models’ predictability; all extractable OM fractions were positively correlated, indicating that an increase in organic matter content also increases the amount of HWC, EOC, and EON. We may expect that other EOM fractions behave similarly, since most of them are strongly correlated with total C or N (Gianello & Bremner, 1986; Sharifi et al., 2007; Schomberg et al., 2009).

4.4.4 Hot water and CaCl₂ extractable OM

The HWC fraction has been characterized using $^{13}$C-NMR spectroscopy and pyrolysis field ionization mass spectroscopy, and it largely consists of carbohydrates and N-containing compounds, in particular amino-N and amides (Leinweber et al., 1995). These compounds are present in soil solution, loosely associated with minerals or within structures of humified SOM, and therefore partly available for microorganisms. Part of the carbohydrates may also originate from microbial biomass; it can account for maximally 40% of the C extracted by hot water (Balaria et al., 2008). Most of the EOM fractions have been shown to be available for microorganisms. For example, Gregorich et al. (2003) showed that 67 to 84% of the organic C in a hot water extract decomposed during 42 days of incubation. Assuming a C-to-N ratio around 12 (Gregorich et al., 2003), we find that the cumulative mineralized N in our soils accounts for approximately 70% of the amount of N in the hot water extract. This suggests that the extracted OM is approximately equal to the biologically available N pool, and probably functions as a source of N. Since the contribution of hot water extractable OM to N mineralization is not quantified yet, further work is needed to quantify its turnover and to confirm its function as a source of N.

Remarkably, the content of HWC is often highly correlated with total C across different soils and agricultural land uses (e.g. Sparling et al., 1998; Kubat et al., 2008; Spohn & Giani, 2010) and sometimes does not change during incubation of soil (Uchida et al., 2010). The relationship between hot water EOM and mineralizable N may therefore also reflect the size of the biomass which size is usually correlated with both HWC and mineralizable N (Sparling et al., 1998). This may explain why a large proportion of the extracted organic matter was...
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resistant to acid hydrolysis (Curtin, 2006), suggesting that a significant part has a relatively recalcitrant nature. Indeed, others showed that the constituents of hot water extractable OM are heterogeneous in age and turnover rates (Gregorich et al., 2003; Von Lutzow et al., 2007). Differentiation of recalcitrant and bio-available forms of N within hot water EOM would presumably improve its sensitivity as an index of labile soil N, but further work is needed to determine how this can be best achieved. Its value as an index of (potentially) mineralizable N will not be proven until the relationships between its characteristics and in situ soil processes are clearly demonstrated.

In contrast to HWC, all OM fractions extractable with 0.01 M CaCl₂ were significantly smaller than the amount of N mineralized (Table 4.1), indicating that they have to be replenished from SOM to function as a source of N for micro-organisms. Recent evidence indicates that the turnover rate of in situ dissolved or CaCl₂ extractable OM was not associated with the net N mineralization rate (Ros et al., 2010c). A major proportion of this fraction is indeed characterized by high molecular weight and low turnover rates (Appel & Mengel, 1998; Jones & Knielland, 2002; Jones et al., 2004). Nevertheless, EOM fractions extractable with CaCl₂ were significantly correlated with (potentially) mineralizable N, as shown in our experiment (Fig 4.2.A) and numerous other incubation and field studies (reviewed in: Appel & Mengel, 1998; Ros et al., 2011b). Consequently they reflect either total OM, being the leftover of decomposition, or another OM pool actively involved in N mineralization like biomass. The quantification of the N turnover of EOM fractions is still a challenge, but use of ¹⁵N isotope tracing may be helpful here (Ros et al., 2010b).

4.4.5 Use of mineralizable N as a measure of soil N supply

We used mineralizable N, determined as the cumulative net N mineralization over 140 days, as a measure to distinguish between soils with different OM characteristics and N mineralization kinetics. Use of the net N mineralization may underestimate the soil N mineralization capacity because inorganic N consumption occurs simultaneously with its production. Nevertheless, both processes also occur in the field and it is ultimately the net N production that defines what can
be taken up by plants. Highly significant relationships between net N mineralization (determined in anaerobic experiments or aerobic experiments with and without leaching) and N uptake from non-N fertilized plots are reported in numerous studies (reviewed in Keeney, 1982), showing that net N mineralization is a valid relative measure of the soil's ability to release N for plant growth.

However, biological laboratory and chemical extraction methods detect a certain ‘potential of available N’ whereas the realization of this potential under field conditions depends on the actual conditions for mineralization in the specific year. Combination of in situ measurements and predictive modeling to estimate the actual mineralizable N (based on EOM fractions and climatic conditions for example) seems to be the way forward to achieve accurate estimates of plant available N under field conditions. A better understanding of the mineralization and immobilization reactions under varying climatic conditions is therefore required. This merits further investigation but history shows that a successful approach, where the N needs for plant growth can be satisfied by mineralizable N, requires a better understanding of the basic concepts involved and better management of the plant soil system (Nannipieri & Eldor, 2009).

4.5 Conclusion

In spite of 70 years of effort, one of the major goals of N research, that of being able to predict net N mineralization, potentially mineralizable N and fertilizer N needs, has not been adequately achieved (Nannipieri & Eldor, 2009). Multivariate statistical modeling has not been applied on this research field, as far as we know, but it allowed us to combine soil variables into two groups relevant for determining mineralizable N: one reflecting the OM content of the soil and another reflecting texture-related characteristics. However, organic matter variables were far more important than any other soil variable. We identified that the amount of C released during hot water extraction gave the best representation of the OM group in our data set. Other EOM fractions have also been identified as the best predictor of mineralizable N (reviewed in: Ros et al., 2011b), suggesting that the most appropriate EOM fraction differs among data sets, possibly
reflecting differences among regions, years, seasons, land uses, and variations in the analysis reproducibility of extraction methods. Combining different OM fractions into one model is not likely to give a robust estimate of mineralizable N due to strong multi-collinearity. These findings suggest that the search for the best predictor of (potentially) mineralizable N can not be answered by statistical evaluation of relationships between EOM fractions and (potentially) mineralizable N alone. We need to know the underlying mechanisms before we can identify the most appropriate EOM fraction. Its value as an index of (potentially) mineralizable N will not be proven until the relationships between its characteristics and in situ soil processes are clearly demonstrated.

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CHAPTER 5

ISOTOPIC ANALYSIS OF DISSOLVED ORGANIC NITROGEN IN SOILS

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Abstract

Determination of the isotopic signature of dissolved organic nitrogen (DON) is important to assess its dynamics in terrestrial ecosystems. Analysis of \(^{15}\)N-DON, however, has been hindered by the lack of simple, reliable, and established methods. We evaluate three off-line techniques for measuring the \(^{15}\)N signature of DON in the presence of inorganic N using a persulfate digestion followed by micro-diffusion. The \(^{15}\)N-DON signature is calculated from the difference between total dissolved \(^{15}\)N (\(^{15}\)N-TDN) and inorganic \(^{15}\)N. We quantified the \(^{15}\)N recovery and signature of DON, NH\(_4\), and NO\(_3\) in a series of inorganic N/DON mixtures (with a TDN concentration of 10 mg N l\(^{-1}\)) for three lab protocols. Phenylalanine was used as a model compound for DON. The best lab protocol determined the concentration of inorganic N and TDN prior to diffusion using improved spectrophotometric techniques. An accuracy of 88% for \(^{15}\)N-DON should be routinely possible; coefficient of variation was <2.9%. Hence, reliable \(^{15}\)N-DON values are obtained over an DON concentration range of 2.3-10 mg l\(^{-1}\). High levels of DON could influence the accuracy of \(^{15}\)N-NO\(_3\) mainly at DON:NO\(_3\) ratios above 0.4. Evaluation of alternative NO\(_3\) measurements is still necessary. Our method is applicable for soil solution samples and soil extracts and has no risk of cross-contamination. Potential applications are large, in particular for \(^{15}\)N tracer studies, and will increase our insight in DON behaviour in soils.

5.1 Introduction

Dissolved organic nitrogen (DON) plays a key role in the soil nitrogen (N) cycle. Foremost, formation of DON represents a pivotal step in the process of N mineralization (Schimel & Bennett, 2004), consequently affecting nutrient availability in natural and agricultural soils. Direct uptake of DON by plant species can also be a potentially important pathway for plant N uptake and may lead to a “short-circuiting” of the N cycle (Neff et al., 2003). Finally, leaching losses of DON from terrestrial (agro) ecosystems is more substantial than often assumed (Van Kessel et al., 2009). Although most factors affecting DON dynamics are thought to be known (Kalbitz et al., 2000; Ros et al., 2009), their quantitative effect on the production and consumption of DON, and their mutual interaction, is only sketchily known and still widely debated. For example, Kemmit et al. (2008) discussed the contribution of abiotic and biotic mechanisms primarily responsible for the production of active organic compounds, like DON, in soil. In addition, Chen & Xu (2008), reviewing the role of DON in forest ecosystems, concluded that it is still unclear how DON is affected by microbial biomass and biochemical characteristics of DON and how it interacts with soil properties (e.g., clay minerals, organic carbon, pH). Knowledge of the above-mentioned aspects is critical for the sound assessment of the role of DON in N cycling. Stable isotope tracing may be very useful to follow DON transformations in soil (Robinson, 2001), as already shown for the dynamics of inorganic N (Murphy et al., 2003).

DON concentrations are commonly measured using UV oxidation, persulfate digestion, or high temperature combustion (Sharp et al., 2002). Essentially, all these methods measure total dissolved N (TDN) after destruction of organic compounds and calculate DON concentrations by subtracting inorganic N from TDN. Similarly, the isotopic signature of DON can be calculated by correcting the $^{15}$N signature of TDN with the inorganic $^{15}$N signature.

Measurements of $^{15}$N fractions in water samples or soil extracts are often performed using micro-diffusion or distillation techniques (Stark & Hart, 1996; Mulvaney et al., 1997; Mulvaney & Khan, 2001). These methods are principally
based on conversion of aqueous to gaseous N, after which it is trapped as an \( \text{NH}_4^+ \)
salt in an acidic medium. This approach has been used for analysis of \( \text{^{15}N-NH}_4^+ \),
\( \text{^{15}N-NO}_3^- \), and \( \text{^{15}N-TDN} \) in soil extracts, soil solutions, and freshwater samples
(Liu & Mulvaney, 1992; Appel & Xu, 1995; Slawyk & Raimbault, 1995; Seely & Lajtha, 1997; Whalen et al., 1999). However, existing methods have not been
rigorously assessed for analysis of \( \text{^{15}N-DON} \), and issues like interference be-
tween inorganic N and DON (Liu et al., 1996; Mulvaney et al., 1997; Sigman et al., 1997) and cross-contamination (Saghir et al., 1993) were not systematically
addressed. In addition, sample preparation, gaseous losses during diffusion, blank corrections, and incorrect inorganic N analyses may introduce an error in
the determination of the \( \text{^{15}N} \) signature of DON (Stark & Hart, 1996; Sharp et al.,
2002). Other techniques for \( \text{^{15}N-TDN} \) analyses, such as the high-temperature
catalytic oxidation furnace (Huygens et al., 2007), have a relatively high detection limit (20 mg N l\(^{-1}\)) and require low salt levels (<0.1 M). Numerous collected
soil extracts do not meet these requirements, limiting the analysis of the isotopic
signature of DON. Therefore, a straightforward assessment of the role of DON
through \( \text{^{15}N} \) tracer studies is still hampered by the lack of established methodology to measure the \( \text{^{15}N} \) signature of DON.

Here we propose and evaluate a method for measuring the \( \text{^{15}N} \) signature
of DON in the presence of inorganic N. The method is based on \( \text{^{15}N-TDN} \) analy-
sis after a persulfate digestion followed by micro-diffusion and a separate \( \text{^{15}N} \)-
inorganic analysis. Specifically, our aims were to determine (1) the accuracy of
this method with respect to \( \text{^{15}N} \) signature of DON, (2) the best laboratory proto-
cols to reach this accuracy, and (3) the extent of possible interference with inor-
ganic N.

5.2 Materials and methods

5.2.1 Solutions Used

We prepared 10 solutions containing a total of 10 mg N l\(^{-1}\) by dissolving mixtures
of KNO\(_3\), NH\(_4\)Cl, and phenylalanine (as a model compound for DON). Phenylala-
nine was chosen since it has a relatively high resistance to persulfate digestion.
and a moderate sensitivity to interfere in spectrophotometric analyses (Herrmann et al., 2005), while it does not differ in its sensitivity for diffusion compared to other amino acids. We therefore consider it to be an appropriate model compound, which will sooner lead to an underestimation of the method accuracy than to an overestimation. We distinguished two main treatments, one with $^{15}$N-enriched phenylalanine and another with $^{15}$N-enriched inorganic N (with NH$_4$ and NO$_3$ in equal molarities). Enriched N compounds were prepared from 98 atom% $^{15}$N-enriched phenylalanine, 98 atom% $^{15}$N-enriched KNO$_3$, and 10 atom% $^{15}$N-enriched NH$_4$Cl (Campro Scientific GmbH, The Netherlands). The theoretical $^{15}$N enrichment was 10.0 atom% for NH$_4$, 10.18 atom% for NO$_3$, and 10.07 atom% for phenylalanine. For each treatment, solutions containing phenylalanine and inorganic N were mixed, with DON accounting for 0, 25, 50, 75, and 100% of TDN. Treatments were replicated five times. The $^{15}$N enrichment of TDN varied between 0.37 and 10.09 atom%.

### 5.2.2 Spectrophotometric Determination of N Compounds

Initial concentrations of NH$_4$, NO$_3$, and TDN were determined spectrophotometrically (Houba et al., 2000) using a segmented flow analyser (SFA; Skalar). In detail, NO$_3$ was determined after conversion to NO$_2$ by cadmium reduction followed by reduction to a red-coloured diazo compound, after addition of R-naphtylamine and sulfanilamide. The absorbance was measured at a wavelength of 540 nm. Ammonium was determined using the Berthelot reaction in which a phenol derivative (salicylate) formed an indophenol derivative in the presence of NH$_3$ and hypochlorite under catalytic action of nitroprusside. In an alkaline medium, the indophenol derivative has a green-blue colour whose absorbance can be measured at a wavelength of 660 nm. Because the Berthelot reaction is not entirely specific to NH$_4$, interference from organic N compounds, such as amino acids, occurs (Herrmann et al., 2005). To overcome this interference, we injected our sample in an alkaline Borax buffer solution (pH 13) through which the NH$_4$ is converted to NH$_3$. The liberated NH$_3$ is subsequently dialyzed through a gas dialysis membrane and captured in an acid buffer solution (pH 5.2) before addition of the colour reagents for the Berthelot reaction. We
tested this procedure on several synthetic amino acid solutions (threonine, glycine, phenylalanine, glutamic acid, serine, valine, and proline) and found that it strongly reduced the interference between NH\textsubscript{4} and organic compounds; maximally 0.3% of the tested organic compounds was detected as NH\textsubscript{4}, whereas the original SFA method detected 6-31% of the organic compounds as NH\textsubscript{4}. Total dissolved N was determined after digestion of organic compounds by potassium persulfate (K\textsubscript{2}S\textsubscript{2}O\textsubscript{8}). Thereafter, both NH\textsubscript{4} originally present and NH\textsubscript{4} formed by the digestion were converted to NO\textsubscript{3} by K\textsubscript{2}S\textsubscript{2}O\textsubscript{8} oxidation catalyzed by UV radiation. After dialysis, NO\textsubscript{3} was determined spectrophotometrically as described above. Reproducibility of the spectrophotometric analysis of NO\textsubscript{3}, NH\textsubscript{4}, and TDN in our accredited laboratory was earlier determined on multiple analysis of a large set of terrestrial samples according to NEN protocol 7777. The relative reproducibility of the NO\textsubscript{3}, NH\textsubscript{4}, and TDN analysis was 2.0%, 2.2%, and 2.0%, respectively. Detection limit was 0.03 mg l\textsuperscript{-1} for NO\textsubscript{3}, 0.04 mg l\textsuperscript{-1} for NH\textsubscript{4}, and 0.15 mg l\textsuperscript{-1} for TDN.

5.2.3 Definitions
The isotopic signature of NH\textsubscript{4}, NO\textsubscript{3}, TDN, or DON in studies using artificially enriched N compounds (also referred as ‘enrichment’) is usually expressed in the units ‘atom%’ or ‘atom% excess’. The unit ‘atom%’ is the percentage of a specific isotope among the other isotopes of the same element, whereas the unit ‘atom% excess’ expresses the same percentage but then corrected for the natural background signature (natural abundance). The concentration of \textsuperscript{15}N-NH\textsubscript{4}, \textsuperscript{15}N-NO\textsubscript{3} or \textsuperscript{15}N-TDN is the total amount of \textsuperscript{15}N present in the solution in the form of NH\textsubscript{4}, NO\textsubscript{3}, or TDN, respectively. We use the term ‘recovery’ to assess the accuracy of the three protocols; it express the percentage of the added compound (e.g., NH\textsubscript{4}, \textsuperscript{15}N-NH\textsubscript{4}) that is detected with the method used.

5.2.4 Micro-diffusion of Inorganic N and TDN
The isotopic signature of TDN and dissolved inorganic N (DIN) was determined using a micro-diffusion technique modified from Stark & Hart (1996). Glassfiber micro-filters (Whatman, GF/A) of 6 mm diameter were spiked with 13 µl of 2 M
KHSO₄ and packed in Teflon tape (19 mm width) to seal the filter from the solution while enabling diffusion of NH₃. An amount of solution (Vsol) containing 30-100 µg of NH₄ and NO₃ was taken from the mixed stock solutions (see section solutions used) and collected in a 100 ml container. Solid KCl (when Vsol > 50 ml) or a 2 M KCl solution (when Vsol < 50 ml) was added to obtain a 1 M KCl solution, in order to get an ionic strength of the solution which is close to the ionic strength of the acid trap, thereby avoiding swelling and breaking of the trap. To have comparable headspaces in all the samples, the volume of solution was brought to approximately 100 ml with 1 M KCl. Preliminary tests showed that comparable headspaces are desired to get similar diffusion gradients above the trap and to equalize possible N losses in the headspace. The pH was raised to approximately 10 with ~0.4 g of ashed MgO. One packed filter was added per container, after which the cup was closed. Containers were left at 20 °C for 7 days with intermittent shaking every other day. After 7 days, the packed filters were collected, washed in demineralised water, unpacked, and dried. Filters were placed in tin capsules for analyses of total N (includes the liberated and trapped NH₄ from the solution and the contamination from the blank; see Blank Correction) and its ¹⁵N signature. After removal of the filters for ¹⁵N-NH₄ determination, 0.4 g of Devarda’s alloy was added to the solution to convert NO₃ into NH₄. One filter was added per container, after which the cup was closed and stored at 20 °C for another 7 days. Again, samples were analysed for total N and ¹⁵N signature. The isotopic signal of TDN was measured after persulfate destruction of a separate subsample, following a modified method of Cabrera & Beare (1993). Briefly, persulfate reagent (5 g of K₂S₂O₈, 3 g of H₃BO₃, and 1.5 g of NaOH dissolved in 100 ml of water) was added to the solution (ratio 1:1) in a Kimax glass tube (Fisher 14-930-10J), capped immediately, and placed in a water bath at 100 °C for 5 h. After cooling down, a sub-sample was taken to determine NO₃ concentration in order to check whether all organic compounds were digested (using initial TDN as a reference). Subsequently, the pH was raised above 10 by adding 2 ml of 6 M NaOH. After pH adjustment, ¹⁵N-NO₃ was measured after micro-diffusion, as described before (includes conversion to NH₄ and subsequent diffusion).
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Analyses of total N on the filter and its $^{15}$N signature were carried out on an automated C/N analyser-isotope ratio mass spectrometer (ANCA-IRMS, Europa Scientific Integra) at UC Davis Stable Isotope Facility. Instrumental precision based on repeated analyses of 5-10 atom% enriched (NH$_4$)$_2$SO$_4$ standards was better than $\pm 0.23\%$.

5.2.5 Evaluated Lab Protocols

Three off-line lab protocols were tested on their ability to determine the enrichment and concentration of inorganic $^{15}$N, $^{15}$N-TDN, and $^{15}$N-DON. They were in increasing order of complexity (1) the $^{15}$N concentration of the various N pools was calculated from the amount and signature of N diffused on the filter, assuming full diffusion recovery (referred to as filter protocol) (2) the $^{15}$N concentration of the various N pools was calculated from the signature of N diffused on the filter and the initial concentration in the solution prior to micro-diffusion measured by standard spectrophotometric procedures (SFA protocol) (3) the $^{15}$N concentration of the various N pools was calculated from the signature of N diffused on the filter and the initial concentration in the solution prior to micro-diffusion, measured by spectrophotometric procedures after separation of amino compounds and NH$_3$ by a gas dialysis membrane (GD-SFA protocol). In addition, we tested the destruction efficiency of the TDN analysis (to test to what extent DON was converted to NO$_3$), the diffusion efficiency of the micro-diffusion of NH$_4$, NO$_3$, and TDN (to test to what extent NH$_4$ or NO$_3$ were converted to NH$_4$ on the filter), possible interference between inorganic N and DON, and contamination from different N measurements and chemicals used.

5.2.6 Blank Correction

The amount of N contamination from blanks is often quantified by measuring the N content of the filter after diffusing a blank KCl solution. However, Stark & Hart (1996) observed that the recovery of N from blanks was significantly lower than the recovery of diffused samples. They suggested to derive the blank contamination from dilution of isotope standards.

We therefore calculate the mass of N in the blank filter, $M_b$, as
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\[
M_f = \frac{M_{\text{std}} \cdot (E_{\text{dif}} - E_{\text{std}})}{(E_f - E_{\text{dif}})}
\]

where \(M_{\text{std}}\) is the mass of N in the standard, \(E_{\text{std}}\) is the \(^{15}\)N enrichment measured in non-diffused standards, \(E_{\text{dif}}\) is the enrichment measured in the diffused standards, and \(E_f\) is the enrichment of the blank filter. The \(^{15}\)N enrichment of the sample can subsequently be calculated by

\[
E_s = E_{\text{dif}} + \frac{M_f \cdot (E_{\text{dif}} - E_f)}{M_s}
\]

where \(E_s\) is the corrected \(^{15}\)N enrichment of the sample and \(M_s\) is the mass of N in the sample, prior to diffusion.

5.3 Results and discussion

5.3.1 Spectrophotometric Interferences

Ammonium was overestimated by 4-100% due to its interference with phenylalanine when determined with the SFA protocol (see Appendix A4, Fig. A4.1A). Almost 10% of phenylalanine was detected as \(\text{NH}_4\). Other amino compounds have also been shown to interfere with \(\text{NH}_4\) (Herrmann et al., 2005). The amount of amino compounds that is detected as \(\text{NH}_4\) may even increase to 99% (e.g., for threonine). Use of gas dialysis prior to spectrophotometric determination (GD-SFA protocol) strongly increased the selectivity of the \(\text{NH}_4\) analysis; less than 0.3% of phenylalanine was detected as \(\text{NH}_4\) (Appendix A4, Fig. A4.1A). This interference between \(\text{NH}_4\) and amino acids indicates that studies using \(^{15}\)N as a tracer may provide erroneous estimations of the turnover rate of N pools when \(\text{NH}_4\) is determined with the classical Berthelot mechanism. Although amino acid pools in soils are generally small (Jones et al., 2005), they increase when crop residues or manures are applied to soil (Stevenson, 1982). Gross N mineralization rates that have been estimated with classical spectrophotometric \(\text{NH}_4\) analyses may be reduced by 4-33% when accounting for amino acid interference with \(\text{NH}_4\) (Herrmann et al., 2005).
5.2.2 Accuracy and Precision

The accuracy and precision of the three lab protocols for the determination of the total concentration, enrichment, and $^{15}$N concentration of NH$_4$, NO$_3$, TDN, and DON are shown in Table 5.1. An accuracy above 88% for $^{15}$N-DON should be routinely possible over an DON concentration range of 2.3-10 mg l$^{-1}$; coefficient of variation (CV) varied between 1.3 and 2.9%. The detection limit of the $^{15}$N-DON measurement depends on its relative contribution to TDN; for solutions containing only DON, the detection limit is ~0.2 mg l$^{-1}$ N. The filter protocol had significantly higher CV values compared to both the GD-SFA and the SFA protocol, indicating that quantification of diffusion losses improves both the accuracy and the precision of the $^{15}$N-DON analysis.

Table 5.1. Accuracy and precision of three protocols for the analysis of concentration and isotopic signature of NH$_4$, NO$_3$, TDN, and DON

<table>
<thead>
<tr>
<th>N analysis</th>
<th>GD-SFA protocol</th>
<th>SFA protocol</th>
<th>Filter protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CV (%)</td>
<td>Recovery (%)</td>
<td>CV (%)</td>
</tr>
<tr>
<td>NH$_4$ concentration</td>
<td>0.4 – 2.0</td>
<td>100</td>
<td>0.4 – 2.8</td>
</tr>
<tr>
<td>$^{15}$N-NH$_4$ enrich</td>
<td>0.3 – 6.4</td>
<td>101</td>
<td>0.3 – 6.4</td>
</tr>
<tr>
<td>$^{15}$N-NH$_4$ conc.</td>
<td>0.8 – 8.2</td>
<td>100</td>
<td>0.5 – 5.7</td>
</tr>
<tr>
<td>NO$_3$ concentration</td>
<td>0.2 – 0.9</td>
<td>100</td>
<td>0.2 – 0.9</td>
</tr>
<tr>
<td>$^{15}$N-NO$_3$ enrich</td>
<td>0.3 – 30</td>
<td>89</td>
<td>0.3 – 30</td>
</tr>
<tr>
<td>$^{15}$N-NO$_3$ conc.</td>
<td>0.5 – 30</td>
<td>89</td>
<td>0.5 – 30</td>
</tr>
<tr>
<td>TDN concentration</td>
<td>0.4 – 0.9</td>
<td>97</td>
<td>0.4 – 0.9</td>
</tr>
<tr>
<td>$^{15}$N-TDN enrich</td>
<td>0.1 – 2.3</td>
<td>98</td>
<td>0.1 – 2.3</td>
</tr>
<tr>
<td>$^{15}$N-TDN conc.</td>
<td>0.8 – 3.0</td>
<td>94</td>
<td>0.8 – 3.0</td>
</tr>
<tr>
<td>DON concentration</td>
<td>0.5 – 5.4</td>
<td>93</td>
<td>0.8 – 3.3</td>
</tr>
<tr>
<td>$^{15}$N-DON enrich</td>
<td>1.7 – 4.2</td>
<td>95</td>
<td>1.6 – 4.2</td>
</tr>
<tr>
<td>$^{15}$N-DON conc.</td>
<td>1.3 – 2.9</td>
<td>88</td>
<td>1.1 – 2.9</td>
</tr>
</tbody>
</table>

Solutions contain 10 mg l$^{-1}$ TDN, with DON accounting for 0, 25, 50, 75, and 100% of TDN (with NH$_4$ and NO$_3$ in equal molarities). Solutions with 0 N are excluded from the calculation.
5.2.3 Blank Correction

The recovery of N from blanks is often lower than the recovery of N from samples (Stark & Hart, 1996). This difference between blanks and samples can introduce large errors in the calculation of $^{15}$N-DON. Our results showed that these errors were eliminated when the blank contamination was calculated from dilution of isotope standards.

5.2.4 Micro-diffusion of NH$_4$

The new GD-SFA protocol was most accurate in determining total NH$_4$; the difference between theoretical and measured values was on average 0.01 mg l$^{-1}$ (Appendix A4, Fig. A4.1A). In contrast, the SFA protocol overestimated NH$_4$ due to spectrophotometric interference between DON and NH$_4$, in particular when the percentage of DON exceeded 50%. Incomplete diffusion recovery (~78%; Table 5.1) resulted in an underestimation of NH$_4$ for the filter protocol. Similar results are found for the $^{15}$N-NH$_4$ concentrations (see Appendix A4, Fig. A4.1C).

The observed enrichment of NH$_4$ was ~9.7 atom% in all solutions containing $^{15}$N-NH$_4$, and ~0.37 atom% (natural abundance) in all solutions containing $^{15}$N-DON (see Appendix A4, Fig A4.1B). This indicates that phenylalanine and NH$_4$ do not interfere during diffusion. Similarly, other studies report no interference between NH$_4$ and glycine (Herrmann et al., 2005) and between NH$_4$ and glucosamine or glutamine (Saghir et al., 1993a; 1993b). In contrast, some studies observe a decrease in NH$_4$ enrichment when NH$_4$ is diffused together with amino compounds (Saghir et al., 1993a; Mulvaney & Khan, 1999; Herrmann et al., 2005). This decrease in NH$_4$ enrichment has been attributed to decomposition of amino compounds under alkaline conditions (Mulvaney et al., 1997; Mulvaney & Khan, 1999; Herrmann et al., 2005). This is in particular for methods using diffusion periods longer than 7 days and diffusion temperatures above 50 °C (Mulvaney & Khan, 2001). As the notion that DON decomposes under alkaline conditions is only based on indirect evidence, the dilution of labeled $^{15}$NH$_4$ is quantified after diffusion in the presence of high concentrations of unlabeled DON, the observed decrease in NH$_4$ enrichment is not necessarily due to decom-
position of DON. Mulvaney & Khan (1999) for example, diffused a solution containing 300 µg of 1.504 atom% excess NH$_4$ and 10,000 µg of amino acid-N and found that prolongation of the diffusion period led to an increase in the quantity of NH$_4$ and a decrease in its $^{15}$N enrichment. However, we noticed a strong decline in total $^{15}$N recovery in the trap during diffusion, suggesting some loss of N instead of dilution. Hence, alkaline hydrolysis of DON is not necessarily responsible for the observed decrease in enrichment.

5.2.5 Micro-diffusion of NO$_3$

Concentrations of NO$_3$ were accurately estimated with the SFA protocol; the mean difference between theoretical and observed NO$_3$ concentration was <0.01 mg l$^{-1}$. In contrast, the filter protocol overestimated the NO$_3$ concentration due to breakdown of DON, as shown by a diffusion recovery above 100% (~112%, Table 5.1) and the $^{15}$N signature of the N diffused from solutions containing labeled DON (Fig. 5.1). This suggestion is supported by our observation that the de-

![Figure 5.1](image_url)

**Figure 5.1.** Comparison of observed and theoretical $^{15}$N enrichment (atom %) for NO$_3$ after diffusion of a series of mixtures of inorganic N and DON determined with GD-SFA, SFA, and filter protocols: (left) the solutions containing $^{15}$N labeled DON; (right) the solutions containing $^{15}$N labeled dissolved inorganic N (DIN)
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crease in $^{15}$NO$_3$ from unlabeled DON was quantitatively related to the increase of $^{15}$NO$_3^-$ from labeled DON (Appendix A4, Fig. A4.4). Hence, phenylalanine and NO$_3$ significantly interfered during diffusion.

Alkaline decomposition of DON during diffusion is often suggested as a mechanism for a decrease in the enrichment of NO$_3$ when diffused in the presence of DON (Liu et al., 1996; Mulvaney & Khan, 1999). However, we found no significant decomposition of DON due to a pH increase during the first 7 days. Subsequently, the mechanism of alkaline hydrolysis during diffusion of NO$_3$ can only be valid when we assume a strong discontinuity between the first and second week. It is therefore more likely that the decomposition of DON is enhanced by addition of Devarda’s alloy. This would also explain why most studies found significantly more decomposition of DON for solutions diffused with Devarda’s alloy and MgO than for solutions diffused with MgO only (Saghir et al., 1993a; Mulvaney et al., 1997; Sigman et al., 1997). Hence, DON might be more strongly affected by reductive deamination due to electropositive metal ions than by alkaline hydrolysis.

Several methods might be used to reduce interferences between DON and NO$_3$ during diffusion. These methods include the removal of DON from soil solution prior to diffusion or the conversion of NO$_3$ to gaseous N without deamination of amino compounds. Decomposition of DON can be enhanced by alkalinization and pre-incubation at high temperatures (Sigman et al., 1997) or by the use of ninhydrin to convert amino-N to NH$_3$ (Kennedy, 1965; Sigman et al., 1997; Mulvaney & Khan, 2001; Marsh et al., 2003). Conversion of NO$_3$ to gaseous forms can be done by use of spongy cadmium and sodium azide to reduce NO$_3$ to N$_2$O (McIlvin & Altabet, 2005) or by use of bacterial denitrification (Sigman et al., 2001). Because NO$_3$ is relatively stable in soil solution, whereas amino compounds can be easily deaminated or decomposed by micro-organisms, methods removing DON should probably be preferred above methods removing NO$_3$. However, neither approach has been validated for $^{15}$N-DON analysis in soil extracts using micro-diffusion techniques.
5.2.6 Micro-diffusion of TDN

TDN concentrations measured after destruction were accurately determined by the SFA protocol (Appendix A4, Fig. A4.3A). The slight underestimation (~3%) was approximately similar to the reproducibility of the TDN analysis (~2%), indicating that differences between observed and theoretical TDN concentrations were not significant. Similar recovery values (ranging between 87 and 102%, mainly >93%) have been found for other amino compounds and humic acid (Bronk et al., 2000; Hagedorn & Schleppi, 2000; Doyle et al., 2004). Possible N losses during diffusion were small since TDN determined with the filter protocol underestimated the concentration with only 5%. The isotopic signature of TDN was accurately assessed with both protocols (Appendix A4, Fig. A4.3B); the difference between theoretical and observed enrichment varied between 0.02 and 0.28 atom%.

5.2.7 Determination of $^{15}$N-DON

All three lab protocols were able to measure the total $^{15}$N concentration of DON reasonably accurately (Fig. 5.2). The mean recovery of $^{15}$N concentration of labeled DON was 88% for the GD-SFA protocol, 88% for the SFA protocol, and 76% for the filter protocol (Table 5.1). The underestimation of $^{15}$N-DON in the GD-SFA protocol was primarily caused by the overestimation of $^{15}$N-NO$_3$ (on average ~7.2%) due to decomposition of DON during diffusion. Because both DON and NO$_3$ are likely to be enriched in $^{15}$N when enriched N is added to natural samples, and the mean contribution of DON to TDN is approximately 25% (Van Kessel et al., 2009), the underestimation of $^{15}$N-DON in natural samples due to this interference is likely smaller than 6% (estimated from treatment with 25% DON). Nevertheless, an accuracy of 88% is quite high, since it depends on (the accuracy of) six separate analyses; from their reproducibility values we can theoretically calculate that the SE of the mean $^{15}$N-DON concentration varies between 2.0 and 4.3% (95% of the measurements are within the interval $^{15}$N-DON ± 2SE (Ott & Longnecker, 2001).

The micro-diffusion method was able to distinguish labeled DON from unlabeled inorganic N, and unlabeled DON from labeled inorganic N (Fig. 5.2).
Isotopic analysis of dissolved organic N

Figure 5.2. Comparison of observed and theoretical concentrations of DON (A), the isotopic signature (B), and $^{15}$N-DON concentration (C) determined by micro-diffusion of a series of mixtures of inorganic N and DON. Three lab protocols are evaluated: the SFA, the GD-SFA, and the filter protocol. Left, the solutions containing $^{15}$N labeled DON; right, the solutions containing $^{15}$N labeled dissolved inorganic N (DIN).
Measurement accuracy improved when corrected for the diffusion recovery using initial analyses of NO$_3$, NH$_4$, and TDN (Table 5.1). Consequently, both SFA protocols performed better than the filter protocol. Though the $^{15}$N concentration of DON was similar for both SFA protocols, a comparison of the DON concentration and enrichment between both protocols showed that the underestimation of the concentration counterbalanced the overestimation of its enrichment for the SFA protocol. On the basis of these observations we recommend the use of the GD-SFA protocol instead of the SFA and filter protocol. We found that the decrease in $^{15}$N-NO$_3$ due to decomposition of unlabeled DON was similar to the increase in $^{15}$N-NO$_3$ due to decomposition of labeled DON (Appendix A4, Fig. A4.4), suggesting that the interference between both fractions can be quantified when a similar solution without added $^{15}$N is spiked with labeled NO$_3$. The isotopic signature of the diffused NO$_3$ will then be different from the spiked NO$_3$ due to the initial amount of NO$_3$ present and decomposition of DON. Consequently, decomposition of DON during diffusion can be calculated from the difference between spiked and diffused NO$_3$.

5.2.8 Counterbalancing errors and error propagation

From standard error propagation rules (Taylor, 1982) we find that the accuracy of the DON analysis depends on the concentration of TDN, NO$_3$, and NH$_4$. Assuming a similar relative variance for each measurement, the highest absolute error contribution originates from TDN, since TDN includes all other N fractions. Accurate inorganic N analyses are therefore required; Sharp et al. (2002) compared the accuracy of DON analyses in seawater samples across 29 laboratories around the world and showed that much of the variability in DON was due to inaccurate analysis of inorganic N. Our results showed that the GD-SFA protocol accurately determined the concentrations of NH$_4$ and NO$_3$; recovery was 100% for both fractions (Table 5.1). Using the reproducibility values of the spectrophotometric analysis, we estimated that the standard error of the DON analysis can theoretically vary between 0.20 and 0.25 mg l$^{-1}$ (2-10% of DON) for our treatments (for calculation, see Appendix A4).
Similar to the calculation of the standard error of DON, the SE of the total $^{15}$N concentration of DON, $\delta^{15}$N-DON, can be estimated by the sum of the errors in $^{15}$N-TDN, $^{15}$N-NO$_3$, and $^{15}$N-NH$_4$. Using the reproducibility values for the spectrophotometric and isotopic analyses, $\delta^{15}$N-DON of labeled DON can theoretically vary between 0.01 and 0.02 mg l$^{-1}$ (2-4.3% of $^{15}$N-DON). Note that the accuracy of $^{15}$N-DON analysis also depends on its relative contribution to TDN.

Errors in the N concentration and $^{15}$N enrichment can also counterbalance each other, as shown for the $^{15}$N-NO$_3$ concentration of the filter protocol in the unlabeled DON treatment (Appendix A4, Fig. A4.2). This suggests that the accuracy of $^{15}$N-DON analysis methods cannot only be evaluated using $^{15}$N recovery on the filter, but should include both analyses of enrichment and total N.

5.4. Potential of the method for isotope ecosystem N studies

The micro-diffusion method we propose and have evaluated has the potential to be used for the determination of total $^{15}$N concentration and isotopic signature of DON. It can be applied on both in situ sampled soil-water and extracted soil solutions; we tested this method on soil solution samples collected with centrifugation and lysimeters and on soils extracted with 0.01 M CaCl$_2$, 2 M KCl, and 0.5 M K$_2$SO$_4$. Addition of HgCl$_2$ (40 mg l$^{-1}$) to preserve samples from microbial decomposition did not affect the $^{15}$N-DON analysis. In addition, the micro-diffusion method can be used for samples with relatively small concentrations of DON. When the concentration of DON is < 0.2 mg N l$^{-1}$ (the minimum amount required for our protocols), solutions or filters can be spiked with unlabeled inorganic N as done by Herrmann et al. (2004). Another advantage of the presented methodology is that there is no risk of cross contamination because of the use of disposable 100 ml bottles. In addition, use of Teflon packed traps allows one to track any leakage of NH$_3$ from the headspace; bottles can be incubated in an inverted position, and any leakage of solution can be noted. Finally, these protocols, despite requiring 6-10 days for completion, have the advantage to be relatively simple, inexpensive, and non-toxic.

The methods' main disadvantage involves the interference between DON and NO$_3$ due to the addition of Devarda’s alloy, but this influence on $^{15}$N-DON is
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relatively small in natural samples (estimated at 6%) and can be quantified by spiking a similar but unlabeled solution with labeled N. Separate analysis of $^{15}$N-NO$_3$ using bacterial denitrification or other approaches may further improve the accuracy of this method but needs to be tested on interference with DON.

To conclude, we claim that the evaluated protocol has a high potential and applicability in ecosystem N research, in particular for $^{15}$N tracer studies. It need to be tested whether this protocol can be used in natural abundance $^{15}$N studies. Especially studies focusing on the functional role of DON should benefit from this advance in analytical N isotope research.

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CHAPTER 6

DYNAMICS OF DISSOLVED AND EXTRACTABLE ORGANIC NITROGEN

UPON SOIL AMENDMENT WITH CROP RESIDUES

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Chapter 6

Predicting soil N mineralization

Abstract

Dissolved organic nitrogen (DON) is increasingly recognized as a pivotal pool in the soil nitrogen (N) cycle. Numerous devices and sampling procedures have been used to estimate its size, varying from in situ collection of soil solution to extraction of dried soil with salt solutions. Extractable organic N (EON) not only consists of DON but contains also compounds released from soil biomass and desorbed organic matter. There is no consensus whether DON or EON primarily regulates N mineralisation in soil, and their contribution to N mineralisation has not been quantified simultaneously. We examine the dynamics of three dissolved and extractable organic N pools using $^{15}$N tracing. DON is determined in centrifugated soil solution, and EON is determined in a 0.01 M CaCl$_2$ extract of a field moist or dried soil. The sampling procedure significantly affected the amount, but not the dynamics and origin of the three organic N pools. The DON and both EON pools showed all a significant increase upon crop amendment and returned to their background concentrations within 10 to 30 days. The fraction of DON and EON originating from the crop residue slightly decreased over 138 days and was not different for both pools. The agreement in dynamics, $^{15}$N enrichment and C-to-N ratio’s indicate that dissolved and extracted organic N have a similar role in N mineralisation. Our results also suggest that they make a minor contribution to N mineralisation; changes in the turnover rate of EON were not associated with changes in the net N mineralisation rate.

6.1 Introduction

Dissolved Organic Matter (DOM) plays an important role in chemical, physical, and biological processes in soil, and facilitates the transport of metals, nutrients and organic pollutants in soils. This DOM consists of an array of molecules, generally reflecting the composition of soil organic matter (Zsolnay, 1996). Although the concentration of DOM typically accounts for maximally 2% of total carbon (C) or nitrogen (N) in agricultural soils, it is often considered to be the most dynamic and bioavailable fraction of C (Haynes, 2005; Marschner & Kalbitz, 2003) and the primary source of mineralisable N (Haynes, 2000). It has been used as an estimate of the N supplying capacity of soils (Keeney, 1982; Groot & Houba, 1995; Sharifi et al., 2007) and as an indicator of changes in soil and fertilizer management (Haynes, 2005). Numerous devices and extraction procedures have been used to estimate the soil (solution) concentration of DOM, varying from in situ collection of soil solution to extraction of dried soil by water or salt solutions. The concentration, molecular characteristics, and function of sampled DOM have been shown to strongly depend on the sampling technique (Zsolnay, 2003; Buckingham et al., 2008; Ros et al., 2009).

Being aware of the influence of lab protocols, Zsolnay (1996, 2003) distinguished three functional DOM pools based on a sequential sampling procedure (Fig. 6.1): i) mobile bioavailable DOM collected by leaching of a soil column, ii) immobile, bio-available DOM collected by centrifugation of moist soil, and iii) potentially bio-available DOM collected by mild extraction procedures. Soil extraction without prior centrifugation and leaching results in an Extractable Organic Matter (EOM) pool consisting of all three DOM pools distinguished by Zsolnay (2003) (Fig. 6.1; Kalbitz et al., 2000; Murphy et al., 2000; Ros et al., 2009). This EOM pool contains not only in situ dissolved organic compounds but also compounds originating from soil biomass and soil organic matter since extraction releases organic compounds by cell lysis, desorption, and hydrolysis (Zsolnay, 1996; Ros et al., 2009). The contribution of these additional compounds
is higher when soils are dried before extraction and increases with increasing
extraction intensity (Fig. 6.1; Ros et al., 2009).

Organic N compounds within DOM serve as a N source for micro-
organisms (Haynes, 2005). These organic compounds consist, at least partially,
of easily mineralisable N, and have a major impact on the usually small but rap-
idly cycling N pools (Murphy et al., 2000). Both Dissolved organic N (DON) and
Extractable organic N (EON) are therefore increasingly recognized as pivotal
components of the soil N cycle (Murphy et al., 2000; Chen & Xu, 2008; Van Kes-
sel et al., 2009). Since the bioavailability varies between DON and EON
(Marschner & Kalbitz, 2003; Zsolnay, 2003), their function as a substrate for
micro-organisms likely varies, too. However, the contribution of each of them to
N mineralisation, and their mutual interaction, have not been quantified simul-
taneously.

**Figure 6.1** Methodological relationships among EOM and DOM, biomass, and
their function as nutrient source (arrows denoted by A represent the
view of Schimel & Bennett, 2004, whereas arrows denoted by B repre-
sent the view of Guggenberger & Kaiser, 2003)
There is no consensus whether dissolved or extractable organic N controls the release of inorganic N from soil organic matter. Cookson & Murphy (2004) for example showed that removal of DON by leaching resulted in a significant decline in microbial biomass N, potentially mineralisable N, and gross N mineralisation. Similarly, Marschner & Kalbitz (2003) and Schimel & Bennett (2004) suggested that organic compounds have to be released into the soil solution before they are available to micro-organisms and plants. This suggests that in particular the in situ dissolved organic N (DON, determined by leaching or centrifugation) plays a significant role as N source for micro-organisms (Fig. 6.1, arrows denoted by “A”). In contrast, Guggenberger & Kaiser (2003) suggested that sorption of DON into bio-films at the solid surface is a prerequisite for mineralisation. Since the bio-films are present on and adsorbed to the surface of soil particles, the bio-available fraction of DON is mainly associated with the weakly adsorbed DOM pool (Fig. 6.1; arrows denoted by “B”). Similarly, Kalbitz et al. (2000) suggested that the formation of DON depend on the decomposition rate of EON. The DON in soil solution is then the left-over of incomplete decomposition of recalcitrant soil organic matter (Hagedorn et al., 2004) and not a main N source for micro-organisms. This would also suggest that extraction of soil with weak salt solutions releases more bio-available organic N than in situ collection of soil solution. Hence, it is unknown which pool has to be collected when one is interested in a pool that primarily serves as a N source for micro-organisms.

The origin and function of extractable organic N have been studied using incorporated $^{15}$N-labeled crop residues (Appel & Xu, 1995; Appel et al., 1996a; Steffens et al., 1996). Use of $^{15}$N labeled residues allows one to follow the fate of residue and soil-derived N fractions in soil. Amending soils with crop residues usually increases both DON (Chantigny, 2003; Van Kessel et al., 2009) and EON (Steffens et al., 1996; Appel & Mengel, 1998; Chantigny, 2003), probably due to the presence of soluble compounds in the amendments. These soluble compounds rapidly decompose and both DON and EON return to their original, background concentrations. However, possible differences between the dynamics of DON and EON after crop residue amendment have not been investigated yet. Isotope tracing of $^{15}$N in EON has also been used to provide evidence whether
EON was actually mineralised and to assess the selectivity of extraction methods to release bio-available organic N (Kelley & Stevenson, 1985; Appel & Xu, 1995; Steffens et al., 1996). To which extent EON or DON contribute to N mineralisation needs still to be investigated.

**Objectives**: in this study we evaluate three sampling techniques on their ability to extract bio-available organic N and their ability to detect temporal changes in these organic N pools due to N mineralisation in soil. The three procedures include the determination of DON in 1) soil solution by centrifugation, and the determination of EON in 2) a 0.01 M CaCl$_2$ extract of field moist soil, or in 3) a 0.01 M CaCl$_2$ extract of dried soil. We refer to ‘moist EON’ and ‘dried EON’ when it is necessary to distinguish between last two sampling techniques. In particular, we investigate how these organic N pools change after incorporation of crop residues. We added $^{15}$N-labeled crop residues to create a temporarily increase in dissolved and extractable organic N, to stimulate microbial activity, and to test whether the source and dynamics of the three pools differ.

We hypothesize that EON initially contains more organic N from the crop than DON, because solid crop particles may release organic N during extraction while these additionally released compounds extraction are not transported to the soil solution yet; transport to the soil solution is only possible by diffusion. In addition, a higher diffusion gradient between soil and extractant compared to the gradient between soil and soil solution promotes additional release of organic N. Furthermore, soil drying and subsequent rewetting may destroy cell walls of the residues releasing organic N to the extractant. At last, because the contribution of biomass-N is higher for EON than for DON (Fig. 6.1), immediate immobilisation of crop-N will result in a higher increase of EON compared to DON. If this hypothesis is correct, then we expect an initially higher $^{15}$N enrichment and $^{15}$N content in EON than in DON after incorporation of $^{15}$N-labeled crop residues. We also expect a higher concentration for EON than for DON during the whole experiment because EON partly originates from desorption of soil organic matter and lysis of the microbial biomass. In agreement with the suggestions of Zsolnay (1996), we hypothesize that DON has a higher turnover rate than EON. We therefore expect DON to decrease faster than EON after crop residue
amendment. In addition, since EON and DON are supposed to intermediate in N mineralisation, we expect that changes in the N mineralisation rate of crop residues are associated with changes in the turnover rate of EON and DON.

6.2 Material and methods

6.2.1 Soil and crop residues used

Soil was sampled two times by bulking random cores taken with a 4 cm diameter auger of 0-30 cm depth of a loamy sandy arable field in Wageningen, the Netherlands. Samples were sieved moist (<5 mm) and plant material and visible organisms were removed by hand. A sub-sample was oven-dried at 40 °C, sieved (<2 mm) and analyzed for initial soil characteristics. The pH of the soil was 5.5, total N was 0.15 g kg⁻¹, total C was 1.7 g kg⁻¹, and initial inorganic N varied between 6.5 (experiment 1) and 9.2 mg kg⁻¹ (experiment 2). Labeled rye grass (*Lolium perenne* L.) was grown in a greenhouse during 36 days by fertilization with 25 mg K₁⁵NO₃ per kg soil. Grass clippings were dried at 70 °C for 24 h, and ground (<1 mm). The ¹⁵N enrichment of the ryegrass clippings was 18.4%. Total N and total C was 24.3 and 422 g kg⁻¹, respectively. Leek (*Allium porrum* L.) shoot residues were harvested from a field experiment in 2008, and chopped into 5 mm pieces. Leek residues were not dried before use. Total N and total C was 42.1 and 455 g kg⁻¹, respectively.

6.2.2 N mineralisation experiments

We performed two experiments, using ¹⁵N-labeled rye grass clippings in the first and unlabeled leek residues in the second experiment. Soils were adjusted to 60% of the water holding capacity, and incubated with or without crop residues. The treatment with crop residues received 1.9 g dried grass residues or 27.3 g fresh leek residues (~2.0 g dried) per kg field moist soil. Soil and soil-crop mixtures were incubated in gas-permeable plastic bags (800 g soil per bag; Audiothene 0.10 mm, Art. No. A 15100) at 20 °C and destructively sampled after 0.5, 1, 2, 5, 8, 12, 19, 26, 33, 40, 77, and 120 days (experiment 1) or after 0.5, 1, 2, 5, 7, 13, 21, 48, and 90 days (experiment 2). Incubation treatments were replicated.
two times. Total organic C, total dissolved N (TDN), NH$_4$ and NO$_3$ were analyzed at each time according to the three lab protocols described below. The isotopic signature of inorganic N and DON were determined using a method encompassing persulfate digestion followed by micro-diffusion (Chapter 5).

6.2.3 Sampling protocols for analysis of DON and DOC

The DOM pool was collected using a modified centrifugal drainage technique from Giesler & Lundström (1993). Soil solution (~60 ml) was released by centrifuging 600 g field moist soil for 11 minutes at 4 °C and 7000 rpm in a Sorvall RC 15C plus centrifuge. Two EOM pools were collected using the extraction procedure of Houba et al. (2000). Shortly, inorganic and organic N were extracted from field-moist or oven-dried (40 °C, 24 h) soils by shaking 8.00 g of soil with 0.01 M CaCl$_2$ at 20 °C, at a soil-solution-ratio of 1:10, for 2 h, followed by centrifugation and filtration (0.45 μm). We refer to these pools as the ‘moist EOM’ and the ‘dried EOM’ pool. All soil solutions and extracts were analyzed for pH, total organic C (TOC), TDN, NH$_4$ and NO$_3$. Concentrations of organic N and C in the soil extracts were expressed in mass per volume soil solution, using the moisture content determined by soil drying at 105 °C.

6.2.4 Chemical analyses

Initial concentrations of NH$_4$, NO$_3$, and TDN were determined spectrophotometrically using a Segmented Flow Analyzer (SFA, Skalar, The Netherlands) (Houba et al., 2000). Total DOC was measured using an automatic carbon analyzer (SFA, Skalar, The Netherlands). The isotopic signature of inorganic N and TDN was determined using a micro-diffusion technique modified from Stark & Hart (1996); the signature of DON was subsequently calculated from the difference between $^{15}$N-TDN and inorganic $^{15}$N (Chapter 5). Total NH$_4$ and NO$_3$, and TDN trapped on the filter and its $^{15}$N enrichment were analyzed using an automated C/N analyzer-isotope ratio mass spectrometer (ANCA-IRMS, Europa Scientific Integra, UK) at the UC Davis Stable Isotope Facility.
6.2.5 Statistics

Repeated measures analysis of variance (ANOVA) with sampling technique as main effect (between subjects effects) and the concentrations of DOC or DON over time (n = 11 time steps for experiment with ryegrass, n = 8 time steps for experiment with leek) as repeated measurements (within subject effects) was applied to test whether sampling technique had a significant effect on the size of the dissolved and extractable C and N pools, their C-to-N ratios, and their \(^{15}\)N concentration and signature after addition of crop residues. Tukey’s post hoc comparisons were used to test whether they differ among the three sampling techniques. Tests were done separately for the treatments ryegrass, leek residues, and both control soils. When time had a significant influence on the tested variables (using the Greenhouse-Geisser correction when the sphericity assumption was not met), then we quantified the significance of the changes using a simple contrast test (the initial value was used as a reference). We used the GLM procedure Repeated Measures ANOVA as implemented in SPSS. Differences were considered statistically significant at \(\alpha < 0.05\).

6.3 Results

6.3.1 Dynamics of DON and DOC in soil solution

The concentration of DON varied between 2.9 and 9.6 mg l\(^{-1}\) for the control soil (data not shown) and between 1.7 and 23.4 mg l\(^{-1}\) for the soil-leek mixture (Fig. 6.2). Slightly lower values were found for the experiment with ryegrass residues: DON concentrations varied between 1.6 and 6.3 mg l\(^{-1}\) for the control soil and between 2.0 and 19.0 mg l\(^{-1}\) for the soil-grass mixture. Addition of crop residues increased DON with maximally 15 mg l\(^{-1}\) for leek \((P < 0.05)\) and 13 mg l\(^{-1}\) for ryegrass \((P < 0.001)\). Because ryegrass clippings were dried and ground before addition, the increase in DON due to crop amendment started immediately after incorporation. In contrast, DON increased gradually after the addition of fresh leek residues. Afterwards, DON decreased in both experiments to similar concentrations as in the control soil.
The dynamics of DOC were similar to DON although its increase was substantially higher; DOC varied between 27 and 61 mg l\(^{-1}\) in the control soil (data not shown; effect of time: \(P > 0.05\)) and increased to 820 mg l\(^{-1}\) after addition of ryegrass (\(P < 0.001\)) and to 1038 mg l\(^{-1}\) after addition of leek residues (\(P < 0.01\)). Therefore, C-to-N ratios increased from 9 to 43 after addition of ryegrass residues (\(P < 0.001\)) and from 10 to 44 after addition of leek residues (\(P > 0.05\)), and decreased afterwards.

### 6.3.2 Dynamics of EON and EOC extracted from field moist samples

The concentration of EON in field moist extracted soils varied between 5.0 and 8.6 mg l\(^{-1}\) for the control soil (data not shown) and between 5.9 and 24.0 mg l\(^{-1}\) for the soil-leek mixture (Fig. 6.2). Similarly, moist EON varied between 6.5 and 17.7 mg l\(^{-1}\) for the control soil and between 8.2 and 24.0 mg l\(^{-1}\) for the soil-ryegrass mixture. Addition of residues significantly increased the concentration of moist EON (leek: \(P < 0.05\); ryegrass: \(P < 0.001\); Fig. 6.2). After the initial increase moist EON stabilized at 6 to 10 mg l\(^{-1}\), being slightly higher than its concentration in the control soil. Moist EOC in the control soil was relatively constant (effect of time; \(P > 0.05\)) and varied between 87 and 125 mg l\(^{-1}\) (data not shown). Again, the release of moist EOC from crop residues was substantially higher than the release of moist EON (Fig. 6.2). Consequently, the C-to-N ratios increased from 12 to 28 after addition of ryegrass residues (\(P < 0.001\)) and from 11 to 40 after addition of leek residues (\(P > 0.05\)), and decreased afterwards.

### 6.3.3 Dynamics of EON and EOC extracted from dried samples

The concentration of dried EON varied between 21.2 and 33.0 mg l\(^{-1}\) for the control soil (data not shown) and between 25.5 and 48.5 mg l\(^{-1}\) for the soil-leek mixture (Fig. 6.2). Similarly, dried EON varied between 25.4 and 31.3 mg l\(^{-1}\) for the control soil and between 27.1 and 46.3 mg l\(^{-1}\) for the soil-ryegrass mixture. Addition of leek residues maximally increased dried EON with 21.5 mg l\(^{-1}\) for leek (\(P < 0.01\)) and with 16.2 mg l\(^{-1}\) for ryegrass (\(P < 0.001\)). Afterwards, dried EON decreased and stabilized at a concentration of 26 to 30 mg l\(^{-1}\), being slightly higher than the concentration in the control soil. Again, C-to-N ratios increased from 12
Dynamics of DON and EON upon soil amendment

**Figure 6.2** Dynamics of EON and DON, EOC and DOC, and C-to-N ratio’s in a soil amended with leek or ryegrass residues during 50 days of incubation. Error bars denote +1 SE of the mean for n = 2.
to 22 after addition of ryegrass residues ($P < 0.001$) and from 10 to 20 after addition of leek residues ($P > 0.05$), and decreased afterwards.

### 6.3.4 Dynamics of DON compared to EON

The release of organic N was significantly affected by sampling technique; extraction techniques released more organic N than techniques collecting soil water, in particular when soil were dried prior to extraction (Fig. 6.2). The concentration of moist EON was slightly but almost consistently higher than the concentration of DON after addition of ryegrass residues ($P < 0.001$), but no significant differences were found between both pools after addition of leek residues ($P > 0.05$). Similar behaviour of the organic N pools was observed in the control soils (leek control: $P < 0.01$; grass control: $P > 0.05$; data not shown). The concentration of dried EON was always higher than the concentration of DON and moist EON ($P < 0.001$). However, although sampling techniques affect the concentration of EON and DON, their dynamics were remarkably similar.

Addition of crop residues increased the concentration of DON, moist EON, and dried EON ($P < 0.05$). The released organic compounds were rapidly decomposed, and all organic N pools returned to their background concentration within 10 to 30 days (Fig. 6.2); the difference between dissolved or extractable organic N in the soil-residue mixture and the control soil was lower than 2 mg N l$^{-1}$ ($P > 0.05$) at the end of the incubation for all sampling techniques (data not shown). The contribution of the crop residue to DON, moist EON, and dried EON was slightly decreasing over 138 days and not different for the three pools (Fig. 6.3.C; $P > 0.05$).

Similar results were observed for DOC and EOC, with the exception that the size of the absolute increase in DOC due to crop amendment did not differ among the three sampling techniques (Fig. 6.2). Differences between DOC and moist EOC in the control soil were more pronounced than differences between DON and moist EON (data not shown); extraction resulted in significantly more EOC than present in soil solution ($P < 0.001$). Remarkably, the C-to-N ratio from dried EOM was higher compared to the C-to-N ratio of moist EOM and DOM after addition of ryegrass ($P < 0.01$) but not after addition of leek residues.
6.3.5 Mineralisation of soil and crop residues

Inorganic N linearly increased from about 39 mg l\(^{-1}\) to about 210 mg l\(^{-1}\) during 90 days of incubation of the control soil in both experiments (e.g., Fig. 6.4.A). Net N mineralisation rate was therefore 1.9 mg l\(^{-1}\) day\(^{-1}\) for both control soils. We could distinguish three periods differing in mineralisation rates after addition of ryegrass residues. In the first two days after addition of the ryegrass residues, all inorganic N was immobilized. This decrease in inorganic N was associated with a strong decrease in DOC during the same period (Fig. 6.2). The second period (day 2 to day 40) was characterized by net N mineralisation, but residue-N was still immobilized. In the third period, after 40 days, DOC concentrations were back to the levels of the control soil, and both soil-N and residue-N were mineralised. Net N mineralisation rate was 3.3 mg l\(^{-1}\) day\(^{-1}\). Similar results were found when leek clippings were added; all inorganic N initially present was immobilized during the first 13 days (data not shown). Afterwards, inorganic N linearly increased with 4.5 mg l\(^{-1}\) day\(^{-1}\).

6.3.6 Isotope tracing N flows

Addition of \(^{15}\)N-labeled crop residues immediately increased the concentration of \(^{15}\)N-EON and \(^{15}\)N-DON in the soil solution (Fig. 6.3.A). After this initial increase, \(^{15}\)N-EON decreased from 2.1 mg l\(^{-1}\) to 0.4 mg l\(^{-1}\) after 138 days when analyzed on dried soil. It decreased from 0.7 mg l\(^{-1}\) to approximately 0.1 mg l\(^{-1}\) after 40 days when analyzed on field moist soil, whereas \(^{15}\)N-DON decreased from 0.9 to 0.2 mg l\(^{-1}\). Unfortunately, we could not analyze the isotopic signature of DON and moist EON for the two last sampling dates because their concentration was lower than the measurement error in inorganic N (~2%). In contrast to the dissolved and extracted organic N pools, inorganic \(^{15}\)N decreased during the first few days to almost zero and increased afterwards to about 16 mg l\(^{-1}\) (Fig. 6.4.C). The initial decrease in inorganic \(^{15}\)N was associated with a strong decrease in DOC, likely indicating that all inorganic \(^{15}\)N was taken up by the microbial biomass using DOC as an energy source. Consequently, most of the \(^{15}\)N in the soil solution was present in organic form during the first 25 days (Fig. 6.4.D). After
those 25 days, the recovery of $^{15}$N in inorganic N increased whereas the recovery in DON decreased. After 138 days, about 34% of the grass residues was mineralised, mainly being present in inorganic form.

The concentration of dissolved or extractable organic $^{15}$N was significantly affected by the methodology used (Fig. 6.3.A; $P < 0.001$). Drying soils prior to extraction could increase the $^{15}$N concentration with 100 to 400% compared to its concentration in the field-moist extracted soil or soil solution ($P < 0.001$). The difference between the $^{15}$N-DON concentration in soil solution and the $^{15}$N-EON concentration in field moist extracted soil was less pronounced; moist $^{15}$N-EON was on average 0.16 mg l$^{-1}$ higher than $^{15}$N-DON ($P < 0.05$). In spite of the quantitative differences among the three organic N pools, their enrichment seemed not to be affected by the sampling technique (Fig. 6.3.B); the enrichment of DON in centrifugated soil solution, and of EON in a CaCl$_2$ extract of dried and field moist soil were not significantly different during the first 40 days of incubation ($P > 0.05$). The enrichment of those pools started at about 4% and decreased afterwards to 1~3%. The $^{15}$N enrichment of dried EON continuously decreased from 4% to almost 2% whereas the enrichment of inorganic N showed a more variable pattern (Fig. 6.4.B). The enrichment of inorganic N decreased from 10 to 2% during the first 7 days, increased from 2 to 5% during the next 18 days, decreased again to 2.2% during the next 15 days, increased afterwards and levelled off at 4.5%. This pattern was not only present in the in situ soil solution but also in the soil extracts (data not shown). The contribution of residue-N to DON, moist EON, and dried EON was similar (Fig. 6.3.C; $P > 0.05$), showing a slight decrease over time; its contribution varied between 8 and 25% for EON determined on dried soils, 4 and 22% for EON determined on field moist soil, and between 12 and 23% for DON in soil solution collected by centrifugation.

### 6.4 Discussion

The concentration of dissolved and extractable organic N was significantly affected by the sampling technique; CaCl$_2$ extraction released more organic N than sampling of pore water. The most important factor increasing organic N
The $^{15}$N concentration (A), $^{15}$N enrichment (B), and the percentage of N originating from added ryegrass residue (C) for DON, moist EON, and dried EON during 50 days of incubation. Error bars denote +1 SE of the mean for $n = 2$.

Figure 6.3
seemed to be soil drying prior to extraction; its concentration increased with more than 100% after drying (Fig. 6.2). Similar results were found for carbon; EOC concentrations were significantly higher than the DOC concentrations in soil solution. The released organic compounds during drying originate not only from desiccated microbial cells, but they also include exo-cellular polysaccharides, and desorbed or hydrolyzed soil organic matter compounds (Kelley & Stevenson, 1985; Appel & Mengel, 1998; Zsolnay et al., 1999; Haynes, 2005). The isotopic signature of EON measured in dried soils suggests that most of it originates from breakdown of soil organic matter and not from desiccated microbial cells: its enrichment gradually decreases during 138 days (Fig. 6.4.B), while it should increase if most of EON had originated from microbial cells because the biomass had immobilized significant amounts of $^{15}$N (Fig. 6.4.C). Similar results were shown by Appel et al. (1996a) who found that almost no bacterial biomass

Figure 6.4 Cumulative N mineralization after addition of ryegrass residues during 138 days of incubation (A), and the $^{15}$N enrichment (B), the $^{15}$N concentration (C), and the $^{15}$N recovery (D) for both EON and inorganic N. Error bars denote +1 SE of the mean for $n = 2$. 

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was extracted from dried soils when $^{15}$N-labeled bacterial biomass was added prior to extraction.

The concentration of EON differed from DON not only due to soil drying but also due to the influence of the extraction procedure on sorption equilibria. Desorption of organic compounds can significantly affect the concentration of EON, since experimental factors like shaking time, extractant and extraction temperature have been shown to affect the desorption rate of organic N compounds (Reemtsma et al., 1999; Zsolnay, 2003; Jones & Willet, 2006). Consequently, soil extracts only provide a comparative estimate of actual DON. However, this influence was quite small (< 5 mg l$^{-1}$) when a field moist soil is extracted with a weak salt solution such as 0.01 M CaCl$_2$; DON and moist EON were not significantly different after addition of leek residues ($P < 0.95$) whereas field moist extraction after addition of ryegrass residues could release about 6 mg l$^{-1}$ more organic N than present in the soil solution (Fig. 6.2.). This also indicates that the capacity of the soil to buffer DON was low, probably depending on the sandy texture and relatively low organic matter content of the soil used (Kaiser & Zech, 1999; Qualls, 2000). Nevertheless, extraction of soil releases more organic compounds than in situ collection of soil solution, as obviously shown for organic carbon in both control soils (data not shown; $P < 0.001$).

The source (crop or soil organic matter), dynamics and C-to-N ratio’s were generally similar for DON, moist EON, and dried EON (Figs. 6.2 and 6.3) in spite of the quantitative differences between them. All three fractions showed an initial increase directly after incorporation of the crop residues and a fast (ryegrass) or gradual (leek) decline afterwards. In contrast to our first hypothesis, the initial increase of EON was not higher than the increase in DON (Fig. 6.2). In addition, the $^{15}$N enrichment of EON was consistently similar to that of DON (Fig. 6.3.B). These results suggest that decomposing plant litter and microbial cells not necessarily enter EON before they are released in soil solution. It also suggests that these methodological defined pools can not be associated with a specific and different function in N mineralisation (e.g. Zsolnay, 1996). This is supported by the observation that a similar portion of DON, moist EON and dried EON originated from the crop (Fig. 6.3.C). Nevertheless, the enrichment of
EON, in particular moist EON, tends to show more variation over time than the enrichment of DON. This could indicate that the organic matter fraction released by 0.01 M CaCl$_2$ extraction is partly more involved in N mineralisation than the in situ DON, contrasting our second hypothesis. There are no other papers simultaneously quantifying the turnover rates of DON and EON, but research on dissolved and extractable organic C supports our indication that EON may have a higher turnover rate than DON (Wagai & Sollins, 2002; Hagdorn et al., 2004).

The relatively steady enrichment of DON in soil solution indicated that the input of unlabeled and labeled DON was equal during incubation or that this DON pool was not affected by or involved in N mineralisation. It is not reasonable to assume a constant contribution of both soil and crop to the production of DON, because their mineralisation kinetics significantly differ (Fig. 6.4.A). In addition, the mineralisation rate of crop residues increased during the experiment, but the enrichment of DON and EON showed a gradual decrease in time. Since the mineralisation rate of soil organic matter was constant (Fig. 6.4.A; the rate is the change of cumulative N mineralisation over time), this gradual decrease in the $^{15}$N enrichment of DON or EON indicates that there was also no change in their input. Hence, these observations suggest that changes in the turnover rate of DON and EON are not associated with changes in the N mineralisation rate of crop residues. This suggestion is supported by the observation that the enrichment of DON, moist EON, and dried EON was lower than the enrichment in inorganic N during main part of the incubation, and even developed in an opposite direction (Fig. 6.4.B); an unlikely result when all organic N is converted in DON before it enters the inorganic N pool. Similar results were observed for EON after the addition of $^{15}$N labeled rape residues (data recalculated from Appel & Xu, 1995; Appel et al., 1996b).

However, since DON and EON are also heterogeneous in their composition (Jones et al., 2004), it could be that one part of these conceptual pools is so rapidly replenished that it is not detected by any one of the sampling techniques we evaluated. Consequently, all these sampling techniques (e.g. centrifugation of soil solution and extraction with weak salts) would collect recalcitrant and
Dynamics of DON and EON upon soil amendment

less bio-available organic N rather than a fraction actively involved in N mineralisation. This indication is corroborated by results of Hagedorn et al. (2004) who showed with $^{13}$C isotope tracing that recently added C was preferentially respired as CO$_2$ without entering DOC; most of DOC was produced during incomplete decomposition of recalcitrant soil C.

Chemical methods extracting organic N from soils have been used to quantify the amount of bio-available N, often assuming a causal relationship between both variables (Griffin, 2008; Ros et al., 2009). Indeed, numerous studies found a significant correlation between initial levels of CaCl$_2$ extractable organic N and net N mineralisation (Groot & Houba, 1995; Appel & Mengel, 1998; Matsumoto & Ae, 2004). This correlation suggests that the size of EON is an indicator of the flux through it (Haynes, 2005): soils with higher levels of EON have higher mineralisation rates. Our results, however, suggest that a causal relationship does not exist; changes in the turnover rate of EON were not associated with changes in the net N mineralisation rate of crop residues (Fig. 6.4.A; change of the cumulative N mineralisation over time). Similar indications were given by Appel & Mengel (1993). A statistically significant relationship between EON and bio-available N therefore suggests that they are simultaneously affected by another variable. More research is necessary to reveal the mechanistic processes behind the interactions among DON, EON, and bio-available N.

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CHAPTER 7

UNRAVELING THE FATE OF DISSOLVED ORGANIC NITROGEN IN SOIL

Gerard H. Ros
Willem H. van Riemsdijk
Chapter 7  Predicting soil N mineralization

Abstract

Extractable and dissolved organic N (EON, DON) fractions are increasingly recognized as important components of nutrient cycling in soil-plant ecosystems. The aims of this study are to: i) investigate the fate of DON (fractions) in soil N mineralization using $^{15}$N tracing, ii) examine the importance of abiotic and biotic processes controlling DON, and iii) examine its relation with extractable, biomass, particulate, and mineral associated N. Extractable organic N fractions are obtained with CaCl$_2$, K$_2$SO$_4$, and hot water. The dynamics and isotopic signature of aforementioned N fractions are studied during a long term (129 days) aerobic incubation experiment where $^{15}$N labeled crop residues have been applied. Residue application released a pulse of biodegradable and recalcitrant DON that temporarily dominated the soil DON and EON pools. The majority of DON and EON (> 80%) was derived from soil organic matter and was comprised of recalcitrant compounds. Their isotopic signature differed from microbial biomass and mineralized N likely due to fast (minutes to hours) decomposition and sorption. As a consequence, current sampling techniques collect a DON fraction that is comprised of (intermediate) decomposition waste products rather than of highly bio-available N compounds. The heterogeneous composition of DON limited the application of isotope tracing techniques, and additional simulation modelling is necessary to quantify the contribution of DON fractions to N mineralization.

7.1 Introduction

Soil organic matter (SOM) strongly affects soil fertility and biomass production in terrestrial ecosystems. Surface soils commonly contain between 0.1 and 0.8% total N, almost entirely in the organic form. If 1 to 3% of this N is mineralized in a growing season (Keeney, 1982), from 20 to 450 kg N ha\(^{-1}\) may be available for crop uptake. Accurate assessment of this soil N supply is therefore an important component of cost-effective, environmentally sound nutrient management in agriculture (Velthof et al., 2009). To develop a method that accurately estimates this soil N supply has therefore been a goal of scientists in past decades, and a number of chemical and physical fractionation methods have been proposed (Nannipieri & Eldor, 2009; Ros et al., 2011b). Essentially, all these fractionation methods aim to isolate chemically labile from recalcitrant, or physically active from protected organic matter, where the chemically labile and physically active fraction may represent the biologically available fraction of SOM (Wander, 2004). The SOM fractions usually proposed include particulate organic matter (POM), dissolved organic matter (DOM), extractable organic matter (EOM), and microbial biomass C and N.

All these four SOM fractions are significantly involved in N mineralization. Particulate organic matter consists of partially decomposed fresh organic matter (e.g., plant litter), and it acts as a substrate and centre for microbial activity, a short-term reservoir of nutrients, and a food source for soil fauna (Gregorich et al., 2003). Dissolved organic matter consists of organic compounds dissolved in the soil solution and it represents the bottleneck in N mineralization because organic matter has to become dissolved before it can be taken up by microbes (Chapin et al., 2002; Schimel & Bennett, 2004). Extractable organic matter consists of organic compounds that are extractable or hydrolysable with water or salt solutions and it originates from both living and non living SOM. It may also represent a potential DOM pool that is able to pass into the soil solution under realistic soil conditions (Kalbitz et al., 2000), subsequently reflecting the soils’ potential to supply N (Ros et al., 2011b). Microbial biomass is the living
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microbial part of the soil and ultimately, it is this part that is responsible for the transformation and cycling of organic matter.

In spite of their significant role in N mineralization, their value as predictor of soil N supply has been criticized due to their heterogeneous composition and their dynamic behaviour in soil (Von Lützow et al., 2007; Ros et al., 2009; 2010c). In particular, there is no established evidence that dissolved and extractable organic N (DON, EON) fractions are more actively involved in N mineralization than the remaining soil organic matter. Hence, the reason that SOM fractions often reflect the potential of soils to supply N is still unknown. This lack of biochemical understanding may also explain our inability to explain the contrasting evidence from studies statistically evaluating the relationship of EON fractions with soil N supply. Understanding the basis behind the observed statistical relationship may help to identify the conditions under which the DON and EON fractions can be used to improve fertilizer recommendations, or to design better methodologies.

My previous work suggests that dissolved and extractable organic N fractions made a minor contribution to N mineralization: we showed with $^{15}$N tracing techniques that the size and isotopic signature of dissolved and CaCl$_2$ extractable organic N fractions are relatively unaffected by changes in N mineralization (Ros et al., 2010c). However, since both dissolved and CaCl$_2$ extractable organic N are heterogeneous in composition (Jones et al., 2004; Matsumoto et al., 2004), it is possible that a part of these organic N pools is so rapidly replenished that it is not, or only partly detected by the sampling techniques that have been used to obtain them. Consequently, the contribution of DON to N mineralization may be underestimated. Isotopic tracing of $^{15}$N applied in biodegradable and recalcitrant fractions of DON may indicate whether these fractions are mineralized with a different rate. As far as we are aware, no studies have combined isotopic studies of recalcitrant and biodegradable organic N fractions to understand the fate of DON in N mineralization.

This study examines the fate of DON in soil N mineralization upon soil amendment with crop residues. $^{15}$N labeled radish residue is added to create a
new source of DON, to stimulate microbial activity, and to examine the source of
the measured DON and the flow of N through DON. Measured DON is split into
a biodegradable and recalcitrant fraction using a short term incubation assay,
and the size and isotopic composition of both fractions is followed over time. The
content and isotopic signature of particulate organic N, biomass N, mineral asso-
ciated organic N, and three EON fractions (obtained with CaCl$_2$, K$_2$SO$_4$, and hot
water) has been measured to examine their contribution to the production of
DON, and subsequently to N mineralization.

### 7.2 Material and methods

#### 7.2.1 Soil and crop residues

Soil was sampled in Autumn 2009 by combining random cores taken with a 4 cm
diameter auger of 0-30 cm depth of a loamy sandy arable field in Wageningen,
the Netherlands. Samples were sieved moist (<5 mm) and plant material and
visible organisms were removed by hand. A sub-sample was oven-dried at 40 °C,
sieved (<2 mm) and analysed for initial soil characteristics. The pH of the soil
was 5.5, total N was 1.47 g kg$^{-1}$, total C was 17.8 g kg$^{-1}$, and initial inorganic N
was 25.5 mg kg$^{-1}$, mainly in the form of NO$_3$. Labeled radish (Raphanus sativus
subsp. Oleiferus) was grown in an ongoing field experiment being fertilized with
enriched K$^{15}$NO$_3$ for 60 days. Produced shoots were harvested after two months,
chopped in pieces, oven dried at 70 °C, and grinded (<1 mm). The C and N con-
tent of the crop residues was 371 g C kg$^{-1}$ crop and 17.6 g N kg$^{-1}$ crop, respective-
ly, the water soluble inorganic N content was 0.36 g N kg$^{-1}$ crop, water soluble
organic N was 7.5 g N kg$^{-1}$ crop. The $^{15}$N enrichment of the radish residues was
9.25 atom%.

#### 7.2.2 Incubation experiment and treatments

A long-term aerobic incubation method is used to determine the fate of PON
(particulate organic N lighter than 1.8 g cm$^{-3}$), DON, EON, and inorganic N in
soil. About 100 g of field moist soil (sieved < 5 mm) is moistened till 60% of the
maximum water holding capacity, and incubated for 129 days at 20 °C in gas
permeable plastic bags (Audiothene 0.10 mm, Art. No. A15100). Three treatments are used: 1) a control soil without addition of crop residues; 2) a soil with incorporated $^{15}$N labeled residues, and 3) a quartz sand with incorporated $^{15}$N labeled residues (including an initial addition of microbial biomass). This last treatment has been used to create a more simplified ‘model-system’ since the enrichment of organic N fractions is in this case not diluted by unlabeled soil organic matter. In the treatments with crop residues, the soil has been amended with 2.44 g dried residue per kg dried soil (~43 mg N kg$^{-1}$ soil). Triplicate bags were destructively sampled after 1, 2, 6, 9, 15, 21, 27, 35, 42, 55, 64, 77, and 129 days, and the soil was analysed for its pH, moisture content, particulate organic C and N fractions, dissolved organic C and N fractions, extractable organic C and N fractions, biomass C and N, and inorganic N (see chemical analyses). The cumulative production of CO$_2$ was determined from regular flux measurements on samples incubated in jars under the same conditions as the bag experiment.

7.2.3 Sampling protocols for DOM, EOM, POM, and biomass

Dissolved NH$_4$, NO$_3$ and TDN in the soil solution were analysed in soil solution samples collected with a centrifugal drainage technique modified from Giesler & Lunström (1993). Soil solution (~60 ml) was obtained by centrifuging 600g field moist soil for 11 minutes at 4 °C and 7000 rpm in a Sorvall RC 5C centrifuge. Moisture content before and after centrifugation was determined. The biodegradable and recalcitrant fraction of DOC and DON were determined using a 21-day soil solution incubation assay where DOC and DON disappear-
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tions extractable with CaCl₂ were collected from field-moist or oven dried (40°C, 24 h) soils by shaking 8.00 g of soil with 80 ml 0.01 M CaCl₂ at 20°C followed by centrifugation and filtration (0.45 μm). EOC and EON fractions extractable with hot water are collected from oven dried (40°C, 24 h) soils by shaking 3.00 g soil with 30 ml distilled water for 16 hours in a hot water bath at 80°C, followed by centrifugation and filtration (0.45 um). EOC and EON fractions extractable with K₂SO₄ were determined on field moist soil after extraction with 0.5 M K₂SO₄ for 2 hours at a soil-to-solution ratio of 1:5 followed by centrifugation and filtration (0.45 μm). Biomass C and N were determined using the fumigation-extraction method (Joergensen & Brookes, 2005). Particulate organic C and N were collected using a fractionation protocol modified from Sohi et al. (2001). Particulate organic matter was collected after shaking a soil sample with NaI (density 1.8 g cm⁻³; soil-to-solution ratio 1:4) for ~60 seconds, followed by sonification for 15 minutes, centrifugation for 15 minutes (3000 rpm) and vacuum filtration over a 1.6 μm filter. This procedure was repeated two times to ensure that all POM was removed from the soil. The retained material in the filter was rinsed with 0.01 M CaCl₂, dried at 70 °C, and analyzed for total C and N. The remaining sample was used to determine the mineral associated organic C and N. After removal of POM, the sample was shaken with 0.01M CaCl₂ for 20 minutes after which the CaCl₂ was removed (using centrifugation at 3000 rpm for 10 minutes). This process was repeated two times to ensure complete removal of all NaI from the sample. The soil sample was dried at 70 °C after cleaning, and analysed for total C and N. Concentrations of organic N and C in the soil extracts are expressed in mass per volume soil solution (moisture content determined by soil drying at 105 °C).

7.2.4 Chemical analyses

Concentrations of NH₄, NO₃ and TEN were determined spectrophotometrically using a Segmented Flow Analyzer (SFA, Skalar, The Netherlands) (Houba et al., 2000; Ros et al., 2011a). Total EON and total DON was calculated as the difference between total extractable (or total dissolved) and inorganic N. Total EOC was measured using an automatic carbon analyser (SFA, Skalar, The
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Netherlands). The production of CO$_2$ was determined using a Innova 1312 photo-acoustic infrared gas analyser (LumaSense Technologies AIS, Ballerup, Denmark). The $^{15}$N isotopic signature of biodegradable and recalcitrant DON, EON, and biomass N were determined using a micro-diffusion technique (Ros et al., 2010b). All $^{15}$N measurements, including the $^{15}$N isotopic signature of PON and mineral associated N, and the total C and N determinations of PON and mineral associated organic matter were analysed with an isotope ratio mass spectrometer (ANCA-IRMS, Europe Scientific Integra, UK) at UC Davis.

7.3 Results

7.3.1 Carbon and nitrogen mineralization in soil

The decomposition rate of soil organic C in the control soil decreased over time from 0.46 to 0.10 mg CO$_2$ per hour (Fig. 7.1; inset). Cumulative C-CO$_2$ produced was 787 mg kg$^{-1}$ soil over 129 days, indicating that about 4.4% of the soil C has been respired. Residue addition increased the decomposition rate up to 1.78 mg CO$_2$ per hour and subsequently the cumulative CO$_2$ evolution up to 1160 mg C-CO$_2$ kg$^{-1}$ soil. Calculated as the difference to the control soil, 42% of the C added
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has been respired. This respired C may originate from the decomposition of the added crop residues but also from soil organic matter (priming effect).

Inorganic N concentrations in the control soil linearly increased from 6.7 to 231 mg l\(^{-1}\) during 129 days (Fig. 7.1.A). Net N mineralization rate was therefore on average 1.74 mg l\(^{-1}\) day\(^{-1}\). Addition of radish residues strongly increased the immobilization rate of N initially and hence, inorganic N levels strongly decreased within one day and remained approximately constant until day 27. After day 27, net N mineralisation started and inorganic N levels increased with a rate of 3.0 mg l\(^{-1}\) up to 241 mg l\(^{-1}\). About 28% of the added crop N was mineralized, what has been estimated from the \(^{15}\)N enrichment of the inorganic N pool. In spite of the higher mineralization rate in the residue amended soil, there was no difference in the cumulative amount of mineralized N after 129 days (Fig. 7.1.A). Because residue addition significantly increased the cumulative CO\(_2\) production, the similarity in cumulative mineralized N between control and amended soil suggests that part of the mineralized N is maintained in the biomass or adsorbed to the soil matrix. Assuming that the same amount of N is mineralized per unit respired C in both treatments, then approximately 110 mg N l\(^{-1}\) has been immobilized and adsorbed. In other words, the amended soil system is relatively enriched in N. Gaseous N losses are likely negligible compared to the N mineralization rates under the conditions of this experiment (section 7.3.6; Maag & Vinther, 1996; D’Haene et al., 2003).

The \(^{15}\)N enrichment of inorganic N was remarkably constant over time (Fig. 7.2), and varied between 2.5 and 3.2% when determined in a CaCl\(_2\) extract. This suggests an equal contribution of crop and soil derived N to total inorganic N over 129 days. Because the CO\(_2\) respiration rate of the control and crop amended soil became approximately equal after 60 days, this constant \(^{15}\)N enrichment suggests that either the microbial biomass is able to create a time delay between N uptake and N release or that previously adsorbed \(^{15}\)N compounds are released at such rate that it counterbalanced any soil derived decrease in the \(^{15}\)N enrichment of NO\(_3\).
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The concentration (A) and $^{15}$N enrichment (B) of inorganic N determined in the soil solution (collected with centrifugal drainage) and a CaCl$_2$ soil extract. Error bars denote ±1 S.E. of the mean (n = 3).

Figure 7.2. The concentration (A) and $^{15}$N enrichment (B) of inorganic N determined in the soil solution (collected with centrifugal drainage) and a CaCl$_2$ soil extract. Error bars denote ±1 S.E. of the mean (n = 3).

The enrichment of inorganic N in the soil solution tended to be higher than that determined in the CaCl$_2$ extract, in particular during the first 40 days. This suggests an additional release of less enriched (more soil derived) N during extraction. Indeed, the CaCl$_2$ extract contained more NH$_4^+$ than present in the centrifuged soil solution (data not shown). There was no difference in obtained NO$_3^-$ concentrations between both methods (data not shown). The additional released NH$_4^+$ may originate from NH$_4^+$ present in the soil water that is not collected by the centrifugal drainage sampling method (it collects about 45% of the soil water) and from previously adsorbed NH$_4^+$. The additional released NH$_4^+$ accounted for 9 to 12 mg l$^{-1}$ on average in both the control and crop amended soil, but it peaks up to 20 mg l$^{-1}$ (at day 27) in the crop amended soil. The enrichment of the additional released NH$_4^+$ was slightly but not significantly lower (-0.37 ± 0.40 atom%; ± 1 SE) than that of the inorganic N in the soil solution. This indicates that both the inorganic N in the soil solution and the additionally released NH$_4^+$ originate for 28 to 36% from the added crop N.
7.3.2 Dynamics of dissolved organic C and N

The addition of radish residues increased the DOC concentration initially by a factor of two compared to the control (Fig. 7.3). This increase in DOC disappeared almost completely within 30 days. For the rest of the experiment, the DOC content remained constant for both the crop amended soil and the control. Similarly, DON increased immediately after crop amendment. After 21 days, the difference between control and crop amended soil became insignificant.

For both DOC and DON, the biodegradable fraction was small and relatively constant (Fig. 7.3). The biodegradable DOC and DON fraction varied between 10 and 20% for both the control and crop amended soil, and hence, addition of radish residue didn’t increase the relative contribution of the biodegradable fraction. These results indicate that most (> 80%) of the DOC and DON in the soil solution consists of relatively recalcitrant compounds. In spite of its predominant recalcitrant nature, both DOC and DON significantly increased due to crop amendment and gradually decreased during the first 21 days of incubation. These observations indicate that the recalcitrant organic fraction, such as deter-
mined by the soil solution incubation assay, is not inert in the soil. The decrease in the recalcitrant fraction may be caused by (enzymatic) depolymerisation catalyzed by the soil matrix, or by sorption to the soil matrix (section 7.4.2).

Because the isotopic signature of DON is indirectly calculated from TDN and inorganic N, the measurement error strongly increases when the contribution of DON to TDN decreases. We therefore plotted the original TDN and inorganic N (both NH\textsubscript{4} and NO\textsubscript{3}) data to avoid huge error bars complicating visual clarity (Fig 7.4). The difference between TDN and inorganic N is due to DON.

The enrichment of inorganic N in the soil solution was higher or comparable to that of total dissolved N indicating that the enrichment of DON was almost similar to or lower than that of inorganic N (Fig 7.4.C). In more detail, the enrichment of NO\textsubscript{3} was consistently higher than that of TDN. Similar results were found for NH\textsubscript{4}, but only during the first 42 days of incubation. After day 42, NH\textsubscript{4} levels were around the detection limit. The initial enrichment of DON was about 3.3% and it declined to 1.5% within 27 days. Accurate determination of the enrichment of DON after day 27 was not possible due to low DON concentra-
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tions, but the comparison of inorganic N with TDN (Fig. 7.4.C) suggests that its enrichment remained lower than that of inorganic N.

This suggestion is supported by the consistent decrease in the enrichment of NH$_4^+$ when the soil solution sample was incubated over 21 days (Fig. 7.4.A). This decrease was associated with an increase in the NH$_4^+$ concentration (data not shown), indicating that there is production of less enriched NH$_4^+$. This produced NH$_4^+$ can only originate from the DON compounds in the soil solution, because there are no other N compounds in the soil solution with a lower enrichment than NH$_4^+$. This decrease in the enrichment of NH$_4^+$ in the incubated soil solution sample also suggests that the enrichment of the recalcitrant DON fraction was higher than that of the biodegradable fraction. Hence, the contribution of crop N to biodegradable DON was lower than that to recalcitrant DON, suggesting that microbes differentiate between crop and soil derived DON compounds even within the biodegradable fraction (section 7.4.1).

7.3.3 Dynamics of biomass C and N

Initial microbial biomass C was approximately 72 mg C kg$^{-1}$ soil. Microbial biomass C increased considerably during the first two weeks in both control (+66%) and crop amended soil (+178%), levelled off during the next two weeks and showed an enormous increase between day 40 and 80 (>800%; data not shown). Since this second peak was not detected in biomass N, and this peak was present in both the control and soil amended soil, we expect that this second peak is related to a methodological error. Therefore, we discarded these data.

The control soil samples as well as the amended samples contained approximately 18 mg microbial biomass N kg$^{-1}$ soil at the start of the incubation (Fig. 7.3). After a considerable increase during the first week, the microbial biomass N in the control soil decreased from 30 to 21 mg N kg$^{-1}$. Microbial biomass N in the crop amended samples increased to a maximum of 41 mg N kg$^{-1}$ after 15 days, significantly above control, and decreased to control values after 77 days.

The $^{15}$N enrichment of the biomass showed a similar pattern as biomass N indicating a fast growth during the first 15 days partly using crop N as N source; 30% of the biomass N was derived from crop N during day 15 to day 50. After 50
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days, the enrichment strongly decreased indicating that the microbial biomass uses relatively more soil derived N as its N source.

7.3.4 Dynamics of extractable organic C and N
Extraction of soil resulted in additional release of organic N compounds compared to DON (Fig. 7.6): the concentration of EON always exceeded the maximum DON concentration of 5.5 mg N l$^{-1}$ (Fig. 7.3). A CaCl$_2$ extraction of a field moist soil released almost four times more organic N than present in the soil solution. Nevertheless, it showed the same trend over time with a relatively constant DON concentration in the control soil, an initial increase immediately after crop amendment and a (slight) decrease afterwards (Fig. 7.6). Soil drying increased EON by 150 to 700%, but again, crop addition significantly increased the EON concentration after which it decreased over the 129 days of incubation. Similar behaviour was found for EOC and DOC fractions obtained with CaCl$_2$ extraction of field moist and dried soil (data not shown). Organic N fractions extractable with K$_2$SO$_4$ were partly similar to CaCl$_2$, while in the last period it resulted in significantly higher EON concentrations. This latter increase coincide with a decrease in biomass N, suggesting that soil extraction with K$_2$SO$_4$ prefer-
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...tially release (dead) biomass compounds that are not soluble with CaCl₂. Similarly, the initial build-up of a higher biomass in the control soil (Fig. 7.5) is reflected in the increase in the K₂SO₄ extractable organic N fraction. Remarkably, these increases in EON were not observed in the organic C fractions extracted with K₂SO₄. The EOC concentration varied between 260 and 309 mg C l⁻¹ in the control soil and gradually decreased from 441 to 360 mg C l⁻¹ after residue amendment (data not shown). Hence, the C-to-N ratios of these K₂SO₄ extractable compounds decreased from 10 to 7.5 in both the control and amended soil. The concentration of EOC extracted with CaCl₂ was consistently higher than that extracted with K₂SO₄ in both the control and the amended soil (data not shown). Hot water extraction caused the highest release of organic N (Fig. 7.6.B). The concentration of EON obtained with hot water varied between 320 and 450 mg N l⁻¹, showing on average a relatively constant concentration with a slight tendency to decline (Fig 7.6).

Because the relative contribution of the two EON fractions obtained with K₂SO₄ and CaCl₂ to total extractable N strongly decreased after day 27 due to net N mineralization, the uncertainty in the isotopic signature of EON in-

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**Figure 7.6.** Dynamics of extractable forms of organic N in the control soil (A) and in soil amended with radish residue (B). Error bars denote ±1 S.E. of the mean (n = 3).
increased. Therefore, we compared the enrichment of inorganic N with total extractable N whereas the difference between both is caused by EON (Fig. 7.7).

The enrichment of the extractable organic N fractions was lower than the enrichment of inorganic N for all extractable organic N forms (Fig. 7.7). In addition, their enrichment was relatively constant over time or showed a (slight) decline. The enrichment of the organic N fractions extractable with K₂SO₄ and CaCl₂ were not different, suggesting a similar fate in soil N mineralization. The enrichment of hot water extractable organic N was slightly decreasing, and about one third of the enrichment of inorganic N.

### 7.3.5 Dynamics of particulate and mineral associated OM

The initial concentration of particulate organic N (light fraction) in the control soil was 7.9 mg N kg⁻¹ soil (Fig. 7.8). Crop amendment immediately increased the PON fraction to 9.6 mg kg⁻¹ (day 2), after which the concentration of PON fluctuated between 6.0 and 11.5 mg kg⁻¹. Its concentration was on average higher in the amended than in the control soil. These observations suggest that most

![Figure 7.7](image_url)

**Figure 7.7.** The enrichment of extractable forms of organic N (A) after amended with labeled ¹⁵N radish residue. Part B compares the enrichment of inorganic N with the enrichment of total extractable N for N fractions extractable with K₂SO₄, CaCl₂, and hot water. Error bars denote ±1 S.E. of the mean (n = 3).
of the added crop N was mineralized or adsorbed within two days of incubation: the higher PON content after crop amendment accounted for only 3.8% of the added crop N. This value approximately corresponds to the recovered $^{15}$N in the PON fraction (Fig. 7.9). The fast decline during the first week indicates that the crop derived PON fraction was quickly mineralized. For the next 121 days, the PON enrichment slightly declined to 0.58%, indicating that in the end of the incubation only 0.3% of the crop N was left in the PON fraction (Fig. 7.9). Similar

**Figure 7.8.** Content of particulate (A) and mineral associated organic N ($N_{ads}$; C) in control soil and in soil amended with $^{15}$N labeled radish residue. The enrichment of both fractions is shown in the right side (B, D).
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behaviour was observed for particulate organic C (data not shown); the C-to-N ratio was on average 17 and decreased from about 18 to 16.5.

As expected, the concentration of mineral associated organic C and N was not affected by crop amendment (Fig. 7.8): the amount of added crop N is negligible (~44 mg N kg\(^{-1}\) soil) compared to the amount of mineral associated organic N (~1000 mg kg\(^{-1}\) soil). However, crop addition significantly increased the \(^{15}\)N enrichment of this fraction from 0.37 up to 0.60 atom% immediately after incorporation. This suggests that more than 50% of the added \(^{15}\)N was present in this mineral associated fraction. The enrichment of the mineral associated organic N fraction remained constant over the rest of the incubation (Fig. 7.8.D), indicating that the crop derived N was not preferentially mineralized.

7.3.6 Mass balance and recoveries of \(^{15}\)N

The contribution of \(^{15}\)N to dissolved, CaCl\(_2\) extractable, and particulate organic N fractions all showed a strong increase in the first week originating from the incorporated crop residue: the percentage crop derived N increased up to 10 to 30% (Fig. 7.9). After this initial increase, the percentage crop derived N strongly decreased to almost zero. The percentage crop derived N in microbial biomass and inorganic N immediately increased up to 20 to 30%, remained constant during the whole experiment for inorganic N and decreased to 15% for microbial biomass N. The contribution of crop N to the mineral associated N was constant at 2% over 129 days of incubation. Remarkably, a major part (37-67%) of the added \(^{15}\)N was found in the mineral associated fraction. During the first 42 days almost no changes occurred in the \(^{15}\)N content of the mineral associated fraction, but it significantly declined when net N mineralization started (Fig. 7.9.C).

Similar behaviour was shown for biomass N, although the change over time was more gradually. About 5 to 17% of the \(^{15}\)N added was present in biomass N. As expected, the recovery of \(^{15}\)N in inorganic N increased over time up to 27%. The amount of \(^{15}\)N recovered was much lower in the extractable, dissolved, and particulate organic N fraction than in inorganic N, biomass N, and mineral associated N. It initially increased up to 5% for extractable organic N, 2.5% for particulate organic N, and 1.2% for dissolved organic N. After this ini-
Figure 7.9. Recoveries of $^{15}$N in biomass N, DON, EON (CaCl$_2$ extractable), PON, and mineral associated N. Recoveries represent the % of the N that is derived from crop N (A, B) or the % of the crop N added (C, D). Error bars denote ± 1 SE of the mean (n = 3).

Initial increase, it decreased for all these fractions and stabilized at a recovery smaller than 1%.
7.4 Discussion

7.4.1 Fate of DON in N mineralization

The $^{15}$N isotopic signature for DON is lower than that of inorganic N over 129 days of incubation, in accordance with our previous experiments with $^{15}$N labeled rye grass (Ros et al., 2010c). Following the basic assumptions of isotope tracing, we would have expected a higher $^{15}$N enrichment in DON compared to inorganic N (Appendix A5). The main assumption includes that biotic and abiotic processes do not differentiate between labeled and unlabeled N. It is evident that this assumption is valid for inorganic N, but the DON fraction is known to be a heterogeneous mixture of organic compounds (Murphy et al., 2000; Ros et al., 2009), each with their own biodegradability and sorption affinity (Kaiser & Zech, 2000; Jones et al., 2004). Hence, the concept of isotope tracing is not directly applicable to heterogeneous organic N fractions. To overcome this limitation, DON has been fractionated in two parts with different biodegradability using a 21-day soil solution incubation assay. Assuming that all organic N has to be converted into DON before it is taken up by the microbial biomass where it is deaminated and released into the inorganic N pool (the ‘direct route mechanism’; Chapin et al., 2002; Schimel & Bennett, 2004), it is evident that the flow of N through the biodegradable fraction is higher than the flow of N through the recalcitrant fraction. As a consequence, the $^{15}$N enrichment of the biodegradable fraction will be higher than that of the recalcitrant fraction and that of inorganic N (Appendix A5).

Our results, however, suggest i) that the enrichment of both DON fractions is lower than that of inorganic N, and ii) that the enrichment of biodegradable DON is lower than that of recalcitrant DON (Fig. 7.3). Under the conditions of the ‘direct route mechanism’, these observations can only be explained by preferential removal of $^{15}$N labeled biodegradable DON from the soil solution. The first mechanism for this preferential removal may be that a certain subfraction of the DON pool is so quickly assimilated or mineralized that it is not detected in the collected biodegradable DON fraction. As a consequence, the majority of the measured DON is comprised of recalcitrant compounds (Fig. 7.3)
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and biodegradable DON becomes less enriched than recalcitrant DON (Fig. 7.4). The second mechanism is principally similar, but in this case is the crop derived DON preferentially removed from the solution by sorption. The fast removal of biodegradable compounds from the soil solution either by sorption or mineralization indicates that the DON that is collected by centrifugal drainage methods is not the only N source for microbes, but it may rather be considered as a mixture of (intermediate) decomposition waste products. Similar conclusions have been made for the fate of dissolved and water extractable organic C in SOM decomposition using $^{13}$C isotope tracing (Guggenberger et al., 1994; Hagedorn et al., 2004; De Troyer et al., 2010).

The above mechanisms explain the striking differences in isotopic signatures of biodegradable DON, recalcitrant DON, and inorganic N, but they are only valid under the conditions of the ‘direct route mechanism’. Recently, Geisseler et al. (2009, 2010) indicated that not only the direct route but also the ‘mineralization – immobilization – turnover (MIT)’ route, in which deamination occurs outside the cell with all N mineralized before assimilation (Barraclough, 1997), is operative in the soil. Exo-enzymatic decomposition may indeed explain the higher enrichment of inorganic N compared to both DON fractions, because of a direct $^{15}$N input from the crop into the NH$_4$ pool. However, it can not explain the lower enrichment of the biodegradable compared to the recalcitrant DON fraction. Similarly, only the isotopic signature of inorganic N can be changed by direct $^{18}$N inputs from previously adsorbed NH$_4$. Nevertheless, because the abiotic and biotic processes simultaneously affect the isotopic signature of inorganic N, it is not possible to exactly quantify the contribution of both DON fractions to N mineralization using the current isotope data. The listed considerations and the limitations of the current isotope tracing techniques emphasize the need for innovative methods determining gross fluxes through DON (e.g., compound specific isotope tracing) and for sampling protocols differentiating between abiotic and biotic mechanisms.

The contribution of the quickly removed $^{15}$N can be estimated from the observed differences in the isotopic signature of inorganic N, biodegradable, and recalcitrant DON (Appendix A5). Using a simplified iterative calculation with
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three DON fractions (a recalcitrant, biodegradable, and a highly biodegradable DON fraction, accounting for 84, 15, and 1% of the total DON, respectively, where the latter DON fraction is fully derived from the crop), I estimate that the turnover time is approximately $4.5 \pm 0.1 \text{ d}^{-1}$ for highly biodegradable DON, $1.6 \pm 0.2 \text{ d}^{-1}$ for biodegradable DON, and $0.08 \pm 0.03 \text{ d}^{-1}$ for recalcitrant DON (Appendix A5). The contribution of these fractions to mineralized N varies between 1 and 42% for recalcitrant DON, between 46 and 87% for biodegradable DON, and between 12 and 13% for highly biodegradable DON. The estimated contribution of the latter DON fraction will increase when these molecules not only originate from crop N but also from native SOM. Hence, these calculations suggest that a significant amount of N is mineralized from another source than the measured DON fractions.

This heterogeneity in turnover rates of DON fractions makes it difficult to assume a causal and positive relationship between the total concentration of DON and the lumped turnover rate of the individual compounds. If such a relationship exists, then it is rather a negative one with increasing concentrations being indicative for lower mineralization rates. Other explanations favouring the contribution of the MIT route or the importance of sorption mechanisms are important to understand the DON dynamics in the soil, but they additionally emphasize that the concentration of DON is not necessarily linked to the production rate of inorganic N.

Simulation modelling of the abiotic and biotic fluxes through the different N pools may be helpful to understand the processes controlling DON and to quantify its relevance for (the prediction of) N mineralization. I developed a simple simulation model based on double Monod kinetics (for microbial growth on biodegradable DOC, DON, and inorganic N), Langmuir kinetics (for sorption of biodegradable and recalcitrant DOM to soil matrix), and first order kinetics (for organic matter decomposition; for a model description, see Appendix A6). At this moment, this model qualitatively describes the observed dynamics in the concentration and enrichment of both DON fractions and inorganic N, supporting our explanation of preferential $^{15}$N removal from DON (Ros, 2011; unpublished da-
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ta). Further work will certainly provide quantitative estimates of the importance of DON fractions in N mineralization over time.

7.4.2 Biodegradability of DOC and DON

Fractionation of DOC and DON shows that the majority of organic compounds (>80%) is present in the recalcitrant fraction (with a lumped turnover time > 21 days). Remarkably, crop addition does not increase the biodegradability of DON (Fig. 7.3). In addition, the recalcitrant DOC and DON quickly respond to crop amendment and gradually decreases to background levels within 21 to 50 days, in spite of its recalcitrant nature (Fig. 7.3).

The relatively constant composition of DOC and DON may be the result of fast turnover and sorption rates within 24 hours after crop amendment. Hence, any change in biodegradable DON has not been detected with the current sampling interval. Fast microbial uptake of biodegradable DON in the soil may explain why the (biodegradable) DOC and DON levels after crop amendment were much higher in the quartz than in the soil treatment during the first 27 days (>1000% higher; data not shown); there was not enough biomass present in the quartz treatment to consume the surplus of biodegradable DON. Sorption of biodegradable DON to the soil matrix may explain why the $^{15}$N enrichment of the mineral associated N fraction strongly increased directly after crop amendment (Fig. 7.9). This may be in the form of organic N (43% of the crop is in water soluble organic form) or in the form of NH$_4$ (being produced before first sampling).

The dynamic fate of recalcitrant DOC and DON suggests that results from soil solution incubation studies are not directly applicable to the situation in the soil. In this experiment, the recalcitrant fraction declines during the first 21 days of the experiment in spite of the fact that it can not be mineralized during a 21-day soil solution incubation assay. This decrease of the recalcitrant fraction in the soil may be caused by (enzymatic) depolymerisation catalyzed by the soil matrix, or by sorption to the soil matrix. Their relatively constant concentration during the second stage of the experiment suggests that they are further decomposed or adsorbed until a new equilibrium is established where the ‘production’ of recalcitrant DON equals its ‘consumption’.
7.4.3 Fate of EON fractions in N mineralization

Extractable organic N fractions obtained with CaCl₂ and K₂SO₄ show a similar isotopic signature (over time) compared to DON, suggesting that the additional released compounds during extraction are similarly controlled by abiotic and biotic mechanisms as DON. This similarity may also reflect a methodological bias for the determination of recalcitrant compounds in soil extractions, because Rousk & Jones (2010) showed that the low molecular weight fraction of EON is almost completely turned over within 15 minutes of extraction where most soil extractions last for 60 to 120 minutes. For these two EON fractions, we may therefore conclude that they are mainly soil derived (Fig. 7.7), consisting of relatively recalcitrant compounds. This conclusion is corroborated by other studies showing with spectroscopic and analytical techniques that EON fractions obtained with salt solutions such as 0.01 M CaCl₂ or 0.5 M K₂SO₄ are characterized by high molecular weight compounds with relatively low turnover rates (Appel & Mengel, 1998; Reemtsma et al., 1999; Jones & Knielland, 2002; Jones et al., 2004). Other studies have equally found a larger proportion of new C in microbial biomass and respired C than in the water extractable organic C fractions (Gregorich et al., 2000; John et al., 2003).

Hot water extractable organic N has been considered as the main N source for mineralization, because its composition is relatively enriched with easily biodegradable compounds (e.g., Landgraf et al., 2006; Balaria et al., 2009), and its size is often comparable to that of (potentially) mineralizable N (Ros et al., 2011a). Most of this EON fraction is derived from the soil (~90%), but it is relatively enriched in ¹⁵N compared to total soil N. The decrease in hot water extractable organic ¹⁵N is almost a factor four lower than the increase in inorganic ¹⁵N, indicating that not all the bio-available ¹⁵N compounds are directly extractable with hot water. Its ¹⁵N enrichment slightly declines over time, being significantly lower than that of biomass N and inorganic N, suggesting that the mineralization rate of crop derived EON was higher than that of soil derived EON (Appendix A5). Differentiation of recalcitrant and bio-available forms of N
within hot water EOM may improve its sensitivity as an index of labile soil N, but further work is needed to determine how this can be best achieved.

7.4.4 Value of EON fractions to predict (potentially) mineralizable N

Chemical methods extracting soil organic N fractions have been used to quantify the amount of (potentially) mineralizable N, often assuming a causal relationship between both variables (Griffin, 2008). The observation that the concentration of DON or EON is not necessarily linked to the production rate of inorganic N does not indicate that their application in soil testing programs is limited. Numerous EON fractions have been shown to correlate with (potentially) mineralizable N (Ros et al., 2011b), and according to this research, this might be explained by an indirect mechanism: an increase in (potentially) mineralizable N is associated with an increase in microbial waste products. The intermediate waste products are further decomposed or adsorbed until a new equilibrium is established with total N. This relationship with total N may explain why all EON fractions were significantly correlated with (potential) N mineralization (Sharifi et al., 2007; Ros et al., 2011b; 2011d). It may also explain why the prediction of (potentially) mineralizable N has been confounded by organic fertilization (Mengel et al., 1999; Smit & Velthof., 2010; Ros et al., 2011b) because organic amendments distort the equilibrium between total N and EON.

7.5 Conclusion

The CO₂ respired and the isotopic signatures of dissolved and extractable N fractions revealed that residue application releases a pulse of biodegradable and recalcitrant DON that temporarily dominates the soil DON and EON pools. The biodegradable DON is quickly removed from the soil solution (< 1 day) after which the recalcitrant fraction declined within 21 days. Beyond that pulse of organic compounds, the concentration and composition of both fractions revert to that of control soils. The majority of DON and EON is derived from soil organic matter and its isotopic signature differs from microbial biomass and mineralized N, casting doubt if these fractions represent the predominant bio-available soil N pool. They may be considered as (intermediate) waste products of organic mat-
ter decomposition, and as such, can they be used to predict (potentially) mineralizable N under steady state conditions.

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CHAPTER 8

GENERAL DISCUSSION

Gerard H. Ros
Chapter 8  
Predicting soil N mineralization

8.1 Introduction

About three-fourth of the organic C contained in terrestrial ecosystems and the majority of organic N is present in plant residues and soil organic matter (Manzoni & Porporato, 2009). Most of the freshly added dead organic matter is mineralized to simple inorganic forms by a highly dynamic community of microbial and faunal decomposers (Bardgett, 2003; Paul, 2007). This supply of macronutrients, in particular for N, has been a topic of considerable research for almost eight decades (Nannipieri & Eldor, 2009; Ros et al., 2011b). The search for a good estimator of this N supply has been the driving force of this thesis.

In this last chapter I discuss the main findings of my research and their implications for the development of soil specific N fertilizer recommendations. The following questions are addressed:

- what is the predictive value of extractable organic N (EON) fractions obtained with chemical extraction methods to predict (potentially) mineralizable N?
- what is the biochemical basis for the relationship between EON and (potentially) mineralizable N?
- is there a future for soil N tests that make use of chemically EON fractions to estimate (potentially) mineralizable N?

8.2 Predictive value of EON fractions

My research has evaluated the possibility to predict (potentially) mineralizable N based on twenty different chemical extraction methods that have been developed during recent decades. The EON fractions obtained through these chemical extraction methods showed a huge variation in size and composition (Chapters 2 and 3) where the EON fraction may account for less than 1% to more than 50% of total soil N. Nevertheless, all twenty EON fractions were significantly and positively correlated with (potentially) mineralizable N with $R^2$ values ranging between 20 and 67% (Chapter 3). The best EON fractions, which are those frac-
tions that can be used to explain more than 60% of the variation in (potentially) mineralizable N, were obtained after soil extraction with acid K$_2$Cr$_2$O$_7$, acid KMnO$_4$, hot CaCl$_2$, and K$_2$SO$_4$. Comparable results are found for EON fractions obtained with cold CaCl$_2$, a phosphate buffer, hot KCl and hot water: they explained on average 46 to 58% of the variation in (potentially) mineralizable N. The significant correlation between EON fractions and (potentially) mineralizable N suggests that EON fractions can be used as a basis for more accurate recommendations of fertilizer N.

However, I also observed that the predictive power of EON fractions to estimate (potentially) mineralizable N strongly varied among studies (Chapter 3), where the uncertainty in the predicted soil N supply can be in the same range as the possible N profit from adjusted fertilizer rates, in particular for the less fertile soils. In addition, the fitted parameter that describes the relationship between EON and (potentially) mineralizable N – indicating how much N is mineralized per unit EON – also varied among data sets that use different sets of soils for their calibration (Chapter 3). The observed variation in this parameter could not be explained with methodological issues alone. Hence, the validity of the calibrated parameter is limited to the conditions of the set of soils used in the calibration experiment thereby seriously limiting the usefulness of fertilizer recommendation systems that are based on such a methodology.

As expected, the predictive power of EON fractions to estimate mineralizable N is significantly lower for mineralization in the field compared to mineralization measured under controlled conditions (Chapter 3). This observation indicates that not only the quality of the organic matter but also environmental factors need to be included to predict N mineralization in the field. Because the situation in the field is the ultimate situation where a fertilizer recommendation system need to be applied, there is the further challenge of relating laboratory incubation data to the variable situation in the field.
Chapter 8

Predicting soil N mineralization

8.3 Is there a biochemical basis behind soil testing?

8.3.1 Overview of the main mechanistic explanations

Several mechanistic explanations have been suggested to account for the positive relationship between EON fractions and (potentially) mineralizable N (Fig. 8.1). These explanations have never been tested or proven to be the reason for the observed correlation. I have studied some of these hypotheses in more detail in my thesis, focusing on organic N fractions extractable with CaCl₂, K₂SO₄, and hot water. Understanding the basis behind the observed statistical relationship may help to identify the conditions under which the EON fractions can be used to improve fertilizer recommendations, or to design better methodologies.

My research suggests that the EON fractions obtained with CaCl₂ and K₂SO₄ are neither the only source of mineralizable N (Chapter 7) nor do they reflect the size of the biomass (Chapters 3), but rather it suggests that the EON fractions can be considered as (intermediate) decomposition waste products in equilibrium with the soil organic matter content (Chapters 4 and 7).

Figure 8.1. Postulated mechanisms explaining the relationship between extractable organic N fractions (EON) and mineralizable N. In the left case, there is a direct link between EON and mineralizable N with EON functioning as its main source. In the middle and right cases, there is no direct relationship between EON and mineralizable N, but their mutual relationship with biomass (middle case) or total soil organic matter (right case) is reflected.
8.3.2 The biochemical basis for DON and EON (CaCl$_2$ or K$_2$SO$_4$)

In more detail, using $^{15}$N tracing I showed that both dissolved organic N (DON) and EON fractions obtained with CaCl$_2$ and K$_2$SO$_4$ may be considered as decomposition waste products (Chapter 7). These waste products may still contribute to N mineralization, but the observed heterogeneity in turnover rates of DON fractions, and the indication that a significant amount of mineralized N originates from a source other than the measured DON fractions, make it difficult to assume a causal and positive relationship between the concentration of total DON and the lumped turnover rate of individual compounds (Chapter 7).

Because the conversion from insoluble to dissolved organic N is considered as the initial and rate limiting step in N mineralization (Chapin et al., 2002; Schimel & Bennett, 2004), my results suggest that most of the labile DON compounds are decomposed before they are widely distributed in the soil solution (Fig. 8.2.). Fast decomposition of labile organic N compounds during soil extraction (< 15 min.; Rousk & Jones, 2010) may explain the similarities between DON and both EON fractions (Chapters 6 and 7). Similar observations have been

![Conceptual diagram of the fate of the biologically available and non-available DON pools. $k_d$ is the (abiotic or biotic controlled) decomposition rate, $k_s$ is the sorption/precipitation rate, and $k_u$ the rate of microbial uptake. The grey box represent the fraction that is predominantly collected by current sampling techniques. The dotted lines represent dead microbial cell inputs, either directly or via faunal grazing.](image-url)
made for the biochemical characteristics of dissolved and water extractable organic C and for their role in SOM decomposition (Guggenberger et al., 1994; Hagedorn et al., 2004; De Troyer et al., 2010).

The decomposition of biodegradable DON in the soil may occur in biofilms at the surface of organic matter particles (Qualls, 2000; Guggenberger & Kaiser, 2003) and is extremely fast with half lives in the region of minutes to hours (Jones & Shannon, 1999; Jones et al., 2005). The striking difference in the $^{15}$N enrichment of the biodegradable DON fraction and inorganic N (with $E_{DON} < E_{inorganic\;N}$) after soil amendment with $^{15}$N labeled residues confirms that a subfraction of the biodegradable DON is so quickly mineralized that it could not be detected in the DON fraction obtained by soil centrifugation (Chapter 7). As a consequence, the majority of the measured dissolved and extractable organic N fractions (>80%) is comprised of less biodegradable compounds (Fig. 8.2). These observations also suggest that the size and composition of the measured dissolved and extractable organic N fractions do not determine how much N can be mineralized. A positive relationship between DON and both EON fractions on the one hand, and (potentially) mineralizable N on the other, may therefore be explained by an indirect mechanism: an increase in (potentially) mineralizable N is associated with an increase in (intermediate) microbial waste products.

8.3.3 The biochemical basis for other EON fractions

Hot water extractable organic N has been considered the main N source for mineralization, because its composition is relatively enriched with easily biodegradable compounds (e.g., Landgraf et al., 2006; Balaria et al., 2009), and its size is often comparable to that of (potentially) mineralizable N (Chapters 3 and 4). Most of this EON fraction was derived from the soil (~90%), but it was relatively enriched in $^{15}$N compared to total soil N (Chapter 7). Nevertheless, it had a slightly declining $^{15}$N enrichment over time, being significantly lower than that of biomass N and inorganic N. Consequently, this EON fraction can only be the main N source for microbes when the mineralization rate of crop-derived EON was significantly higher than that of soil-derived EON (Appendix A5). When the main flow of N occurs through a small sub-fraction of the hot water EON frac-
tion, then it seems unlikely to assume that the size of the total EON fraction is positively and causally related to the net NO$_3^-$ production or soil N supply.

Based on my experiments, it is not possible to make conclusions on EON fractions that are obtained with extraction methods completely different from the ones investigated. However, several years ago, Juma & Paul (1984) determined the selectivity of several more intensive extraction methods (e.g., acid KMnO$_4$, HCl, H$_2$SO$_4$, and hot CaCl$_2$) to obtain a bio-available part of SOM and their results correspond to our observations for hot water extractable organic N. Their results indicate that the EON compounds are not the only source of mineralizable N and they suggest that there is only a remote possibility that a single extractant could extract the variety of N compounds undergoing mineralization and immobilization. The huge diversity of extraction conditions (Chapters 2 and 3) additionally emphasizes the absence of an established relationship between a specific release mechanism and the bioavailability of the released organic compounds, but further research using (double labeled) isotope tracing is necessary to elucidate their biochemical relationship with (potentially) mineralizable N. Simulation modelling of the fluxes through the different pools may also be helpful to quantify their contribution to N mineralization and immobilization (Chapter 7).

The above reasons indicate that the EON fractions obtained with more intensive extraction procedures represent a specific but poorly defined organic matter fraction of the soil. Their size and composition suggests that they cannot be considered as the main food source for microbes or as microbial waste products, and hence, their relationship with (potentially) mineralizable N may reflect their dependency on total soil organic N (Sharifi et al., 2007; Ros et al., 2011d).

### 8.3.4 The role of total soil organic N

When the size and composition of measurable EON fractions do not determine how much N can be or is mineralized, then the positive relationship between EON and (potentially) mineralizable N is likely to depend on their mutual relationship with another soil variable, likely total soil organic matter (Fig. 8.1). This suggestion is corroborated by my observation that both EON and
(potentially) mineralizable N significantly correlate with total soil N (Chapters 3 and 4). It seems evident that an increase in total soil N increases (potentially) mineralizable N, because then there is more substrate available for microbial uptake. Similarly, an increase in the N mineralization will result in an increase in waste products that form the main constituents of both DON and EON (section 8.3.2). These waste products in the soil solution may interact with the soil matrix by sorption mechanisms (Kalbitz et al., 2000; Qualls, 2000), which explains why the concentration of dissolved and water extractable organic C can be modelled with sorption kinetics (Kaiser & Zech, 2000; Vandenbruwane et al., 2007). A strong relationship between EON fractions and total N was also observed in my multivariate analysis of 39 published datasets: measured EON fractions and total organic matter are in more than 95% of the experiments associated within one multivariate model component (Ros et al., 2011e). Consequently, the question ‘how are EON fractions involved in N mineralization’ can be changed in ‘how do both EON and (potentially) mineralizable N depend on total soil organic N’ in order to understand the relationship between EON and (potentially) mineralizable N.

Any disturbance in the relationship between total N and either EON or (potentially) mineralizable N flaws the predictive value of EON to estimate (potentially) mineralizable N. There is indeed some evidence that both EON and (potentially) mineralizable N idiosyncratically respond to environmental conditions (e.g., Miller et al., 2005; Akagi & Zsolnay, 2008) and to soil and nutrient management (e.g., Waring et al., 1994; Curtin & Wen, 1999; Sharifi et al., 2008; 2009). This different response may explain why the correlation between EON and mineralizable N was weaker in fertilized compared to unfertilized soils (Chapter 3). This different response to fertilization also suggests that EON fractions do not reflect the N release from freshly added organic matter (e.g., manures, crop residues), and hence, they predict the magnitude of soil N mineralization rather than that of organic fertilizers. It also indicates that soil samples need to be taken before the first fertilizer application in order to obtain an accurate estimate of the soils potential to supply N.
Because a change in EON due to an external disturbance (e.g., ploughing, fertilization) usually diminishes within a few weeks to months (e.g., Chantigny, 2003), it is primarily the variation in (potentially) mineralizable N that may be responsible for the strong variation observed in the predictive value of EON fractions (section 8.2). This encourages the quantification of factors controlling the relationship between total N and (potentially) mineralizable N. These factors may include environmental controls, cropping history and fertilizer management, since they have significant effects on (potentially) mineralizable N (e.g., Sharifi et al., 2009; Bregliani et al., 2010).

8.4 Is there a future for soil N testing?

8.4.1 Evaluation of the ‘conventional’ soil testing approach

Current N fertilizer recommendation systems (in the Netherlands) differentiate between crop species and soil texture, but account only to a certain extent for other local soil characteristics, in spite of the fact that they significantly affect the potential of the soil to supply N (Chapter 3, Zebarth et al., 2009). The number and variety of chemical extraction methods and generic simulation models mirror a relentless effort to describe and quantify the complex nature of soils and the elemental cycling within them. In spite of 80 years of effort, none of these approaches have been able to adequately predict N mineralization and fertilizer N needs at the farm-scale (Olfs et al., 2005; Nannipieri & Eldor, 2009; Ros et al., 2011b).

The search for an EON fraction that can be used as a basis for predicting (potentially) mineralizable N is usually done by comparing the predictive power of distinct EON fractions. According to my research, the exact EON fraction used is less important (Chapters 3 and 4) and practical considerations may be decisive (e.g., reproducibility, analysis costs, etc) to select one for routine application in soil analysis. I also showed that the combination of different EON fractions does not provide extra information on the amount of mineralizable N compared to one single EON fraction (Chapter 4).
Recalling the limitations related to the use of EON fractions to predict (potentially) mineralizable N (section 8.2), and the variation in soil N supply (section 8.3), I conclude that the ‘conventional’ soil testing approach, which uses linear relationships between EON and (potentially) mineralizable N without any covariates, has to be adapted to improve N fertilizer recommendations.

8.4.2 Improving the soil testing approach

Soil EON fractions reflect the potential of soils to supply N, but the current uncertainty of the predicted soil N supply (even under controlled environmental conditions) is still too big for serious improvement of fertilizer recommendations (Chapter 3). This uncertainty can be related to the idiosyncratic response of EON fractions and soil N supply to, for example, a change in climatic conditions, land use, and soil and nutrient management (Hadas et al., 1986; Groot & Houba, 1995; Sharifi et al., 2008; 2009; Dessureault - Rompré et al., 2010). One obvious need is therefore to quantify the response of both EON and the soils' potential to supply N to the aforementioned issues and to investigate their dependency on total N using multivariate statistics. If grouping of soils or the addition of covariates does not improve the statistical relationship of EON and soil N supply, then this may suggest that the relationship is robust across such groupings and covariates. This robust relationship may be attractive from a practical point of view, but it also suggests that the current uncertainty in the predicted soil N supply reflects a fundamental limitation in our ability to predict soil N supply. Hence, it indicates that a chemically EON fraction cannot account for all the complex interactions between biological, chemical, and physical factors that control N mineralization.

Observations from my multivariate analyses (Chapter 4, Ros et al., 2011e) suggest, for example, that grouping of soils among texture classes may further improve the relationship of EON fractions with (potentially) mineralizable N. Soil texture has indeed been shown to influence N mineralization: course textured soils have a more active microbial population and organic matter is more available for mineralization than soils of fine texture (Hassink, 1992). However, the possible improvement by grouping soil textures is mainly based on evidence
from experiments (including my own) that are dominated by course to medium
textured soils. Therefore, this suggestion needs to be tested on a set of soils
equally distributed among the different textural classes.

The actual N supply in the field strongly depends on environmental condi-
tions, and hence, there is also an obvious need to combine the ‘conventional’ soil
testing approach with simulation models that account for temperature and mois-
ture dynamics during the growing season. The combination of soil testing with
simulation modelling may bridge the gap between the attempts made by
‘conventional’ soil testing and generic simulation models (section 1.2). The
strong point of the soil testing approach is that it gives us not only specific inform-
ation of the quantity and probably also quality of the organic matter present,
but also information on the textural class, acidity, and the size and activity of
the microbial biomass. This relatively static information becomes more valuable
when combined with simulated temperature and moisture data over the growing
season (e.g., Campbell et al., 1997) since these environmental changes have been
identified as important variables in determining N mineralization under field
conditions (Zebarth et al., 2009).

The potential of soils to supply N, and the actual N mineralization, varies
significantly among and within fields (Zebarth et al., 2009). Despite the recogni-
tion of significant spatial variation in optimum fertilizer N rates within fields
(e.g., Snyder et al., 1996; Babcock & Pautsch, 1998), uniform applications of N
are still the norm, resulting in over-fertilization in some parts of the field and
under-fertilization in others (Fiez et al., 1994; Kitchen et al., 1995). This reflects
our limitations to characterize the spatial distribution of soil N supply. Quantita-
tive knowledge of its spatial distribution can be used to identify management
zones within individual fields based on the observed or expected variation in soil
properties. These management zones may be delineated using soil maps, inten-
sive soil sampling, topographic features or crop information from former years
(Olfs et al., 2005; Zebarth et al., 2009).

Hence, a holistic approach, which considers spatial and temporal variabil-
ity of both soil N supply and crop N demand, may provide a more successful ap-
Chapter 8  Predicting soil N mineralization

approach to improving fertilizer management at the farm-scale. A decision support system integrating the aforementioned aspects can be an efficient and effective way to modify fertilizer N management during the growing season in response to fluctuating crop and environmental conditions.

8.5 Conclusions and outlook

My research has contributed to our understanding of the use of soil N tests to predict N mineralization in soils. Foremost, it shows that almost all EON fractions that have been tested have the potential to be used in fertilizer recommendation systems. It also strongly emphasizes that the search for ‘the best soil test’ is not finished by the identification of a soil test with a high predictive value. The dependency of the soil N supply on methodological and environmental issues strongly encourages more effort to be put into validation and up-scaling, particularly regarding the quantification of the differences between laboratory and field experiments. Integrating soil testing with simulation models that account for these differences will evidently improve our ability to predict soil N supply.

My research also clarified that EON fractions obtained with weak hydrolyzing salt solutions reflect the total organic matter content of the soil rather than a bio-available N pool within SOM. Similar mechanism may explain the relationship between potentially mineralizable N and EON fractions obtained with stronger hydrolyzing salt solutions. Looking back on my PhD research, I admit that these conclusions could have been made from i) the existing concept that the quality of soil organic matter is not always the predominant factor controlling N release, even under controlled environmental conditions (Marschner & Kalbitz, 2003; Ekschmitt et al., 2005) and from ii) the observation that most EON fractions have various molecular recalcitrance and physical accessibility to microbial decomposition, bearing in mind that decomposition rates may range from minutes to years (Haynes, 2005; Von Lützow et al., 2007). Indeed, the release of N from soils is controlled by both abiotic and biotic processes, and their
complex interaction with the organic matter in the soil is unlikely to be integrated into the size of one chemically determined EON fraction.

Nevertheless, my study is the first one to quantitatively evaluate the overall predictive value of EON fractions using the meta-analysis approach in which all chemical extraction methods developed during the last 100 years are included. This approach allows me to come up with quantitative and more generally applicable conclusions compared to the results of single experiments. Use of multivariate statistics allowed me to address the issue of collinearity among EON fractions and total N, an issue which the implications of are often overlooked. Lastly, this study is one of the few addressing the biochemical basis behind the relationship between EON and (potentially) mineralizable N in order to understand under which conditions this relationship can be applied.

Based on my observations, I suggest that future research should focus on the understanding and quantification of soil N supply rather than on the development of extraction methods. Relevant research items may include fundamental questions dealing with the importance of SOM quality in relation to environmental, (micro)-biological or soil dependent factors controlling N release, and the spatial heterogeneity of N mineralization in relation to soil structure. They may also include more applied scientific items such as the quantification of the (integrated) effect of temperature and moisture dynamics on N mineralization, the effect of repeated drying-rewetting events, the integration of methods that quantify the N release from both soils and applied organic fertilizers, and the development of methods that can be used to create different fertilizer management zones within fields.

Ultimately, there will always be a level of uncertainty regarding N fertilizer requirements, even if we expand our soil testing efforts to include measures of both inorganic N, (potentially) mineralizable N, and integrated simulation modelling. It might even be that there are fundamental limits to our predictions due to the complexity of mineralization and immobilization processes in soil (Crawford et al., 2005). However, this level of uncertainty does not negate the value of soil N testing, nor should we abandon soil testing for predicting N ferti-
lizer requirements. Soil testing clearly shows measurable fractions of soil N that somehow reflect the soils’ potential to supply N and this information can be valuable when planning fertilizer N applications. However, we need to be realistic about our expectations regarding soil N testing and understand that any estimate of fertilizer N requirements are subject to, among others, the unpredictability of weather.
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Additional info

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APPENDICES

A1. SUPPORTING INFO
Chapter 2

A2. SUPPORTING INFO
Chapter 3

A3. SUPPORTING INFO
Chapter 4

A4. SUPPORTING INFO
Chapter 5

A5. SUPPORTING INFO
Isotope tracing

A6. SUPPORTING INFO
Simulation modelling
Additional info

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### Table A3.1  Correlation matrix for variables used in multivariate PLS modelling (part A, be continued on next page).

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<th>total C</th>
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<th>C-P</th>
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<th>WHC</th>
<th>Initial moisture</th>
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### Table A3.1: Correlation matrix for variables used in multivariate PLS modelling

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<th>P-CaCl2</th>
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<th>EOC moist (% total C)</th>
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### Table A3.1  Correlation matrix for variables used in multivariate PLS modelling (part C).

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<th>Variables (as in Table 4.1.)</th>
<th>HWC (% total C)</th>
<th>EON dried (% total N)</th>
<th>EON moist (% total N)</th>
<th>NO₃</th>
<th>NH₄</th>
<th>Mineral-iz. N</th>
<th>N-to-P ratio</th>
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<td>.000</td>
<td>.003</td>
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Additional info

Predicting soil N mineralization
Abstract

This supporting information contains the theoretical calculation of the standard error of DON and $^{15}$N-DON for the 10 solutions we tested. It also contains 3 figures (Figures A4.1, A4.2, A4.3) with the observed and theoretical total concentration, $^{15}$N enrichment, and $^{15}$N concentration of TDN, NO$_3$, and NH$_4$ for the three off-line protocols we tested. Lastly, it shows (Figure A4.4) that the interference between NO$_3$ and DON can be quantified when a similar but unlabeled solution was spiked with labeled N; the decrease in $^{15}$NO$_3$ due to decomposition of unlabeled DON was similar to the increase in $^{15}$N-NO$_3$ due to decomposition of $^{15}$N-DON.

Error calculation

The concentration of DON is derived from three separate analyses, including total dissolved N, NH$_4$, and NO$_3$, and is calculated by:

$$DON = TDN - NO_3 - NH_4$$  \hspace{1cm} (A4.1)

From standard error propagation rules (Taylor, 1982), we find that the standard error (SE) of DON, $\partial$DON, can be estimated by:

$$\partial DON = \sqrt{(\partial TDN)^2 + (\partial NO_3)^2 + (\partial NH_4)^2}$$  \hspace{1cm} (A4.2)

where $\partial$TDN, $\partial$NO$_3$, and $\partial$NH$_4$ are the absolute SE for TDN, NO$_3$, and NH$_4$, respectively. Reproducibility of the spectrophotometric analysis of NO$_3$, NH$_4^+$, and TDN in our accredited laboratory was determined on multiple analysis of a large set of terrestrial samples according to NEN protocol 7777. The relative SE of the NO$_3$, NH$_4$, and TDN analysis was 2.0%, 2.2%, and 2.0%, respectively. The calculated SE for the tested solutions are shown in Table A4.1.
Similar to the calculation of $\partial$DON, the SE of the total $^{15}$N content of DON, $\partial^{15}$DON, can be estimated by the sum of the errors in $^{15}$N-TDN, $^{15}$N-NO$_3$, and $^{15}$N-NH$_4$. The $^{15}$N-TDN concentration is calculated by:

$$^{15}N_{TDN} = [TDN] * A^%_{TDN} \tag{A4.3}$$

where TDN is the concentration of TDN in the solution and A% is the measured enrichment in atom%.

The fractional error of a quantity is equal to the square root of the sum of the squares of the individual fractional errors (Taylor, 1982). Hence, the SE of $^{15}$N-TDN can be estimated by:

$$\partial^{15}N_{TDN} =^{15}N_{TDN} \sqrt{\left(\frac{\partial TDN}{TDN}\right)^2 + \left(\frac{\partial A^%_{TDN}}{A^%_{TDN}}\right)^2} \tag{A4.4}$$

where $\partial$TDN and $\partial A^%_{TDN}$ are the absolute SE for the concentration and enrichment of TDN, respectively. The SE of $^{15}$N-NO$_3$ and $^{15}$N-NH$_4$ can be estimated similarly. The instrumental precision of the automated C-N analyzer isotope ratio mass spectrometer was estimated on repeated analysis of enriched isotopic

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<th>Concentration [mg l$^{-1}$]</th>
<th>Abs. Standard Error [mg l$^{-1}$]</th>
<th>Rel. SE (%)</th>
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<tr>
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<td>0.200 0.110 0.100 0.249</td>
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</table>
standards. The relative SE of this analysis was found to be 0.11% for samples analyzed at natural abundance levels, 0.11 for 5% enriched standards, and 0.23% for the 10% enriched standards. We used a conservative estimate of 0.23% for the reproducibility values of artificially enriched samples. The theoretical $^{15}\text{N}$ enrichment was 10.0 atom% for NH$_4$, 10.18 atom% for NO$_3$, and 10.07 atom% for phenylalanine. The calculated SE of the $^{15}$N-TDN, $^{15}$N-NO$_3$, and $^{15}$N-NH$_4$ concentration are shown in Table A4.2.

Table A4.2 Calculated SE for the $^{15}$N concentration in TDN, NH$_4$, and NO$_3$ in the 10 tested solutions. Solutions contain 10 mg l$^{-1}$ TDN, with DON accounting for 0, 25, 50, 75 and 100% of TDN (with NH$_4$ and NO$_3$ in equal molarities).

<table>
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<tr>
<th>Solutions tested</th>
<th>$^{15}$N enrichment (atom%)</th>
<th>Abs. SE in [^{15}$N] (mg l$^{-1}$)</th>
<th>NO$_3$</th>
<th>NH$_4$</th>
<th>DON</th>
<th>TDN</th>
<th>$^{15}$N-NO$_3$</th>
<th>$^{15}$N-NH$_4$</th>
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<td>0.3676</td>
<td>10.0679</td>
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<td>-</td>
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<td>0.0154</td>
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<td>0.0002</td>
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<td>-</td>
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When the SE of the $^{15}$N-TDN, $^{15}$N-NO$_3$, and $^{15}$N-NH$_4$ concentration is known, one can calculate the SE of the $^{15}$N-DON concentration using equation A4.2. The estimated absolute and relative SE of the $^{15}$N-DON concentration are given in Table A4.3. When $^{15}$N labeled DON is used, the theoretical relative SE of the $^{15}$N-DON analysis vary between 2.0 and 2.2%.
**Table A4.3** Calculated absolute and relative SE for the $^{15}$N-DON analysis of the 10 tested solutions. Solutions contain 10 mg l$^{-1}$ TDN, with DON accounting for 0, 25, 50, 75 and 100% of TDN (with NH$_4$ and NO$_3$ in equal molarities)

<table>
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<th>Solutions tested</th>
<th>$^{15}$N-DON concentration (mg l$^{-1}$)</th>
<th>Abs. SE of $^{15}$N-DON (mg l$^{-1}$)</th>
<th>Rel. SE of $^{15}$N-DON (%)</th>
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<td>0.001</td>
<td>0.0</td>
</tr>
<tr>
<td>100% unlabeled DON</td>
<td>0.04</td>
<td>0.001</td>
<td>2.0</td>
</tr>
<tr>
<td>75% unlabeled DON</td>
<td>0.03</td>
<td>0.007</td>
<td>24.6</td>
</tr>
<tr>
<td>50% unlabeled DON</td>
<td>0.02</td>
<td>0.013</td>
<td>70.5</td>
</tr>
<tr>
<td>25% unlabeled DON</td>
<td>0.01</td>
<td>0.019</td>
<td>208.1</td>
</tr>
<tr>
<td>0% unlabeled DON</td>
<td>0.00</td>
<td>0.025</td>
<td>0.0</td>
</tr>
</tbody>
</table>
A4. Supporting information chapter 5

Comparison of observed and theoretical concentrations of NH$_4$ (A), the isotopic signature (B), and $^{15}$N-NH$_4$ concentration (C) determined by micro-diffusion of a series of mixtures of inorganic N and DON using the SFA, the GD-SFA and the filter protocol. Left part represents the solutions with $^{15}$N labeled DON; right part represents the solutions with $^{15}$N labeled dissolved inorganic N (DIN).

Figure A4.1
Figure A4.2 Comparison of observed and theoretical concentrations of NO$_3$ (A), the isotopic signature (B), and $^{15}$N-NO$_3$ concentration (C) determined by micro-diffusion of a series of mixtures of inorganic N and DON using the SFA, the GD-SFA and the filter protocol. Left part represents the solutions with $^{15}$N labeled DON; right part represents the solutions with $^{15}$N labeled dissolved inorganic N (DIN).
Comparison of observed and theoretical concentrations of TDN (A), the isotopic signature (B), and $^{15}$N-TDN concentration (C) determined by micro-diffusion of a series of mixtures of inorganic N and DON using the SFA, the GD-SFA and the filter protocol. Left part represents the solutions with $^{15}$N labeled DON; right part represents the solutions with $^{15}$N labeled dissolved inorganic N (DIN).

Figure A4.3
Interference between DON and NO$_3$; the decrease in $^{15}$NO$_3$ due to decomposition of unlabeled DON (y-axis) was similar to the increase in $^{15}$N-NO$_3$ due to decomposition of $^{15}$N-DON (x-axis).

Figure A4.4
A5. Isotope tracing

Abstract

This supporting information contains a mathematical description of the assumptions of isotope tracing, supporting the statements made in Chapter 7. Turnover rates were quantified for i) a homogenous DON pool, ii) a DON pool consisting of two fractions (biodegradable and recalcitrant) and iii) a DON pool consisting of three fractions (highly biodegradable, biodegradable, and recalcitrant).

Estimation of DON fluxes using $^{15}$N tracing

The application of $^{15}$N pool dilution is based on a number of assumptions. These assumptions include ‘no isotopic discrimination’, ‘uniform distribution of $^{15}$N’, and ‘equilibrium between N pools’ (discussed in: Murphy et al., 2003). The first principle assumes that microbial and abiotic processes, which consume N, do not discriminate between $^{14}$N and $^{15}$N isotopes. The second principle assumes a uniform distribution of the applied $^{15}$N label throughout the soil. The last principle assumes that there is an equilibrium between applied and indigenous N pools. Equilibrium in this case refers to the concept that once the $^{15}$N is applied to soil it is in the same chemical state and location within the soil as the indigenous N, and that any soil N transformation process that occur during the subsequent incubation period (e.g., diffusion, fixation, gaseous loss pathways, immobilisation, nitrification) would equally affect the applied and indigenous N pools.

Calculation turnover rates for a homogenous DON pool

The Dissolved Organic N (DON) pool has been considered as the bottleneck for N mineralization: all organic N have to become DON before they are assimilated or mineralized by the microbial biomass (‘the direct route mechanism’; Chapin et al., 2002; Schimel & Bennett, 2004). If we consider a homogenous DON fraction in the soil solution, and we apply $^{15}$N labeled crop N, then all the produced NO$_3$ ultimately originates from decomposition of DON (Fig. A5.1).
Hence, if we denote the flow of $^{14}$N by $B_1$, and the flow of $^{15}$N by $A_1$ (Fig A5.1), then the total mineralized N ((over a certain time interval, $t$)) is:

$A_1 + B_1 = NO_3(t_f) - NO_3(t_0)$  \hspace{1cm} A5.1

The net $^{15}$N flux can be calculated when the NO$_3$ enrichment ($E_{NO3}$) is known:

$A_1 = NO_3(t_f) * E_{NO3}(t_f)$  \hspace{1cm} A5.2

Assuming that biological processes do not discriminate between both isotopes, then flux ratio $^{15}$N/$^{14}$N is similar to the enrichment of DON ($E_D$). Consequently,

$E_D = \frac{^{15}N_{DON}}{^{15}N_{DON} + ^{14}N_{DON}} = \frac{A_1}{A_1 + B_1} = E_{NO3}$  \hspace{1cm} A5.3

Assuming a relatively constant DON concentration and enrichment over time, and combining these equations leads to:

$B_1 = \frac{NO_3}{1 + \left(\frac{E_D}{1 - E_D}\right)} = NO_3 * (1 - E_D)$  \hspace{1cm} A5.4

Figure A5.1  Schematic and simplified overview of the N flow from crop N and indigenous soil organic N to inorganic N via a homogenous DON pool.
If the assumption that biological processes do not discriminate is invalid, then the difference in flux rates of A and B can be estimated by:

\[
\Delta \frac{B}{A} = \frac{1}{E_{\text{NO}_3}} \left( \frac{1}{1 + \frac{E_D}{1 - E_D}} \right) \left( 1 - E_{\text{NO}_3} \right) = \frac{E_{\text{NO}_3} - E_D}{E_{\text{NO}_3}}
\]  

A5.5

It is evident that this assumption can be used for studies using artificially enriched inorganic N, but the DON fraction is known to be a heterogeneous mixture of organic compounds (Murphy et al., 2000; Ros et al., 2009), each with their own biodegradability and sorption affinity (Kaiser & Zech, 2000; Jones et al., 2004). Hence, the concept of isotope tracing is not directly applicable to heterogeneous organic N fractions. To overcome this limitation, I fractionate DON in two parts with different biodegradability using a 21-day soil solution incubation assay (Chapter 7). The first ‘biodegradable’ fraction has a lumped turnover time shorter than 21 days, whereas the second ‘recalcitrant’ fraction has a lumped turnover time longer than 21 days.

**Calculation turnover rates for a DON pool with two distinct fractions**

A schematic and simplified overview of the N flows from crop N and indigenous soil organic N to inorganic N via biodegradable (DON\textsubscript{L}) and recalcitrant DON (DON\textsubscript{R}) is presented in Fig. A5.2. Under the conditions of the ‘direct route mechanism’ it is likely to assume a higher turnover rate for the biodegradable than for the recalcitrant fraction, in particular when fresh organic residues are incorporated.

Similar to that of the homogenous DON pool, the total N and \textsuperscript{15}N mineralization can be calculated by:

\[
A1 + A2 + B1 + B2 = \text{NO}_3(t_x) - \text{NO}_3(t_0)
\]

A5.6

\[
A1 + A2 = \text{NO}_3(t_x) * E_{\text{NO}_3}(t_x)
\]

A5.7
Assuming that biological processes do not discriminate between both isotopes, then the flux ratio $^{15}$N/$^{14}$N is similar to the enrichment of the DON fraction. As a consequence,

For biodegradable DON, with $E_L$ as its enrichment,

$$E_L = \frac{A1}{A1 + B1}$$

for biodegradable DON, with $E_L$ as its enrichment.

For recalcitrant DON, with $E_R$ as its enrichment,

$$E_R = \frac{A2}{A2 + B2}$$

If the DON concentration and enrichment are constant, then the $^{14}$N and $^{15}$N flow from soil and crop into inorganic N via biodegradable DON can be estimated by:

$$B1 = NO_3(t_x) \times \frac{\left( E_{NO3} - \left( \frac{E_R}{1 - E_R} \right) + \left( \frac{E_{NO3} \times E_R}{1 - E_R} \right) \right)}{\left( \frac{E_L}{1 - E_L} \right) - \left( \frac{E_R}{1 - E_R} \right)}$$

Figure A5.2 Schematic and simplified overview of the N flows from crop N and indigenous soil organic N to inorganic N via biodegradable (DON$_L$) and recalcitrant DON (DON$_R$).
A5. Isotope tracing

Subsequently, the N flow via recalcitrant DON is:

\[ B2 = \text{NO}_3(t_L) \times (1 - E_{NO3}) - B1 \]

\[ A2 = B2 \times \left( \frac{E_R}{1 - E_R} \right) \]

Consequently, the turnover rates of biodegradable DON \((T_{DONL})\) and recalcitrant DON \((T_{DONR})\) can be estimated by:

\[ T_{DONL} = \frac{B1 + A1}{C_{DONL}} = \frac{(B1 + A1)}{F \times C_{DON}} \]

\[ T_{DONR} = \frac{B1 + A1}{C_{DONR}} = \frac{(B1 + A1)}{(1-F) \times C_{DON}} \]

where \(C_{DONL}\) is the concentration of biodegradable DON, \(C_{DONR}\) is the concentration of recalcitrant DON, \(C_{DON}\) is the concentration of total DON in the solution, and \(F\) is the relative contribution of biodegradable DON to total DON (value between 0 and 1).

Under the condition of the ‘direct route mechanism’, there is a net N flow from the soil (and crop) in inorganic N via DON. Assuming that the turnover rate of the biodegradable DON pool is higher than the turnover rate of recalcitrant DON, there are the following three boundary conditions:

- \(B1 > 0\),
- \(B2 > 0\), and
- \(T_{DONL} > T_{DONR}\).
If B1 > 0 then it should be that

$$E_L < E_R > E_{NO3}$$ or

$$E_L > E_R < E_{NO3}$$

If B2 > 0 then it should be that

$$E_L > E_{NO3}$$ for the situation that $$E_L > E_R$$

$$E_L < E_{NO3}$$ for the situation that $$E_L < E_R$$

If $$T_{DONL} > T_{DONR}$$ then it should be that

$$F*E_L < E_{NO3} - (1-F)*E_R$$ for the situation that $$E_L > E_R$$

$$F*E_L > E_{NO3} - (1-F)*E_R$$ for the situation that $$E_L < E_R$$

**Contribution of a third ‘undetected’ DON fraction**

Our results, however, suggest i) that the enrichment of both DON fractions is lower than that of inorganic N, and ii) that the enrichment of biodegradable DON is lower than that of recalcitrant DON (Fig. 7.3). Under the conditions of the ‘direct route mechanism’, these observations can only be explained by preferential removal of $^{15}$N labeled biodegradable DON from the soil solution. To estimate the (relative) importance of this ‘undetected’ DON fraction, we distinguish between biodegradable DON (is measured), recalcitrant DON (is measured), and highly biodegradable DON (undetected). Using similar principles as discussed before, we estimate:

Total mineralized N:

$$A1 + A2 + B1 + B2 + A3 + B3 = NO_3(t_o) - NO_3(t_0)$$
A5. Isotope tracing

Total mineralized $^{15}$N:

$$A1 + A2 + A3 = NO_3(t_x) \cdot E_{NO3}(t_x)$$  \hspace{1cm} A5.23

Enrichment of the fluxes:

$$E_L = \frac{A1}{A1 + B1} \quad \text{for biodegradable DON, with enrichment } E_L. \hspace{1cm} A5.24$$

$$E_R = \frac{A2}{A2 + B2} \quad \text{for recalcitrant DON, with enrichment } E_R. \hspace{1cm} A5.25$$

$$E_{SL} = \frac{A3}{A3 + B3} \quad \text{for highly biodegradable DON,}$$

$$\quad \text{with enrichment } E_{SL}. \hspace{1cm} A5.26$$

Assuming a constant DON concentration and enrichment over time leads to:

$$B1 = \frac{NO_3(t_x) \cdot \left( E_{NO3} \left( \frac{E_R}{1-E_R} \right) + \left( \frac{E_{NO3} \cdot E_R}{1-E_R} \right) \right) + B3 \cdot \left( \frac{E_R}{1-E_R} \right) - \left( \frac{E_{SL}}{1-E_{SL}} \right)}{\left( \frac{E_L}{1-E_L} \right) - \left( \frac{E_R}{1-E_R} \right)} \hspace{1cm} A5.27$$

$$B2 = NO_3(t_x) \cdot (1 - E_{NO3}) - B1 - B3 \hspace{1cm} A5.28$$

$$B3 = C \cdot (1 - E_{SL}) \hspace{1cm} A5.29$$

where $A3$ and $B3$ are the $^{15}$N and $^{14}$N flux derived from highly biodegradable DON, and $C$ is the produced NO$_3$ derived from highly biodegradable DON.

The minimum and maximum contribution of this fraction can be estimated for the situation that

$$A1 + B1 = 0, \text{ so that the turnover rate of the biodegradable fraction is 0}$$

$$A2 + B2 = 0, \text{ so that the turnover rate of the recalcitrant fraction is 0}$$
Using the following inputs derived from our observations:

- NO₃ production (observed) = 43.3 mg kg⁻¹
- Eₙₙ₃ (observed) = 0.028 atom%
- Eₗ (estimated from observations) = 0.018 atom%
- Eᵣ (observed) = 0.020 atom%
- Eₛₗ (assumed 100% crop derived) = 0.093 atom%
- DON concentration (observed) = 0.93 mg kg⁻¹
- Contribution recalcitrant DON (observed) = 0.84
- Contribution biodegradable DON (observed) = 0.15
- Contribution highly biodegradable DON (assumed) = 0.01

and the following boundary conditions:

\[ A_1 + B_1 \geq 0; \]
\[ A_2 + B_2 \geq 0; \]
\[ A_3 + B_3 \geq 0; \]
\[ T_{DONL} \geq T_{DONR} \]

I estimated that

\[ 4.16 < B_3 < 5.10 \] (mg kg⁻¹)
\[ 0.00 < T_{DONR} < 0.18 \] with average \( T_{DONR} = 0.14 \) and SE = 0.03 \( (d^{-1}) \)
\[ 0.44 < T_{DONL} < 2.09 \] with average \( T_{DONL} = 1.3 \) and SE = 0.2 \( (d^{-1}) \)
\[ 3.99 < T_{DONSL} < 4.68 \] with average \( T_{DONSL} = 4.4 \) and SE = 0.1 \( (d^{-1}) \)
A5. Isotope tracing
**Figure A6.1** Conceptual diagram with N fluxes as incorporated in our simulation model. Explanation is given in the text. Flow of $^{15}$N is calculated as the flux times the enrichment of the source.
Simulation modelling

I developed a simple simulation model based on Monod equations (microbial growth), first order kinetics (decay of organic matter), and Langmuir kinetics (sorption). This conceptual model is based on the direct route mechanism that stated that all organic compounds have to become dissolved before they can be utilized by micro-organisms. It splits the decomposition/growth process into two stages. The first one is a stage where solid or adsorbed organic compounds are depolymerised and released into the soil solution. This solubilisation step is generally the rate limiting step of decomposition and may depend on abiotic (desorption) or biotic (exo-enzymatic) processes. The second stage is the subsequent utilisation of the substrate to yield new biomass.

The conceptual model distinguish between a highly labile dissolved organic matter pool and a more recalcitrant dissolved organic matter pool since it has evidently been shown that most of the organic N measured in the soil solution is of a high molecular weight and recalcitrant nature. Consequently, organic matter in soil solution must consists of a small pool with a high turnover and a second pool consisting of a more recalcitrant fraction (Chapter 7, Jones et al., 2003). Decomposition of C and mineralization of N are linked through the CN ratio of the biomass and the substrates. The conceptual diagram and the main flows of C and N are presented in Fig A6.1.

Microbial growth

When the supply of a primary substrate is considered to be limiting, microbial metabolism and subsequent microbial growth was assumed to follow the double Monod equation. In the Monod equation the rate of microbial growth with cell decay is described by:

\[
\frac{dC_m}{dt} = \left( u_{\text{max}} * F_C * F_N - K_d \right) * C_m
\]

A6.1
Predicting soil N mineralization

with

\[ F_N = \max \left( \left( \frac{C_{NO3}}{K_{NO3} + C_{NO3}} \right) \left( \frac{C_{BDON}}{K_{BDON} + C_{BDON}} \right) \right) \]  \hspace{1cm} (A6.2)

\[ F_C = \frac{C_{BDOC}}{K_{BDOC} + C_{BDOC}} \]  \hspace{1cm} (A6.3)

where \( C_m \) is the microbial concentration in soil (mg kg\(^{-1}\)); \( C_{BDOC} \) and \( C_{BDON} \) aqueous phase concentrations of biodegradable or labile organic C and N (mg kg\(^{-1}\)), respectively; \( C_{NO3} \) the concentration of NO\(_3\); \( K_{BDOC} \), \( K_{BDON} \), \( K_{NO3} \) the half-saturation constants for microbial growth on labile BDOC, BDON and NO\(_3\) (mg kg\(^{-1}\)), respectively; \( K_d \) the first order endogenous decay coefficient (d\(^{-1}\)); and \( u_{max} \) is the apparent microbial growth rate (d\(^{-1}\)). \( F_C \) and \( F_N \) are the growth limiting factors for C and N, respectively.

**Biodegradable dissolved organic C**

We distinguish between biodegradable and recalcitrant dissolved organic matter since it has been shown that a significant part of the organic matter in the soil solution is highly recalcitrant indicating a relatively low turnover rate and limited bioavailability to micro-organisms. However, some of the recalcitrant compounds may be depolymerized and subsequently used by micro-organisms. Biodegradable dissolved organic C can not only be taken up by microbes but it can also adsorb to the soil matrix being stabilized for further microbial decomposition. Hence the **change in biodegradable DOC** can be calculated as:

\[ \frac{dC_{BDOC}}{dt} = \frac{-C_{up} - C_{ad} - C_{ma} + C_{dep}}{dt} \]  \hspace{1cm} (A6.4)

where \( C_{up} \) is the rate of DOC substrate utilization by micro-organisms (mg kg\(^{-1}\) d\(^{-1}\)), \( C_{ad} \) is the rate of adsorption to the soil matrix (mg kg\(^{-1}\) d\(^{-1}\)), \( C_{ma} \) is the C costs for microbial maintenance (mg kg\(^{-1}\) d\(^{-1}\)), and \( C_{dep} \) is the rate of biodegradable
DOC input originating from depolymerisation of fresh crop residues, dead microbial cells, recalcitrant DOC and native soil organic matter (mg kg\(^{-1}\) d\(^{-1}\)).

The **substrate utilization rate of BDOC** for microbial growth can be expressed using Monod kinetics:

\[
\frac{C_{up}}{dt} = (u_{\text{max}} \cdot F_{C} \cdot F_{N}) \cdot \frac{C_{m}}{Y_{C}}
\]  

where \(Y_{C}\) is the yield coefficient for micro-organisms utilizing C (mass of micro-organism produced per unit mass of substrate consumed (g g\(^{-1}\)). Hence, the **amount of CO\(_2\) produced** is then calculated as:

\[
\frac{dCO_{2}}{dt} = \left( (u_{\text{max}} \cdot F_{C} \cdot F_{N}) \cdot \left( \frac{1}{Y_{C}} - 1 \right) + K_{m} \right) \cdot C_{m}
\]  

The **adsorption of BDOC** to soils can be described with an adapted Langmuir equation:

\[
\frac{C_{\text{ad}}}{dt} = \frac{Q_{\text{max,}\ C} \cdot pC \cdot C_{\text{BDOC}}}{(1 + pC \cdot C_{\text{BDOC}})}
\]  

where \(Q_{\text{max,}\ C}\) is the Langmuir parameter related to maximum adsorption capacity of BDOC to soil particles (expressed as rate, mg kg\(^{-1}\) d\(^{-1}\)), and \(pC\) the parameter related to energy of adsorption (VandenBruwane et al., 2007).

**Carbon inputs into BDOC** originating from dead microbial cells and depolymerisation of fresh and native organic matter can be represented by first order decay of these additional substrates:
Chapter 1  

Predicting soil N mineralization

\[
\frac{dC_{\text{dep}}}{dt} = \left( C_m * K_d + k_s * C_s + k_c * C_c \right) * n + k_{sc} * C_{\text{SDOC}} \tag{A6.8}
\]

where \( C_s \) and \( C_c \) are the concentrations of C in the soil and the amount of C added with fresh crop residues (mg kg\(^{-1}\)), respectively, and \( k_s \) and \( k_c \) the related first order decomposition rates (d\(^{-1}\)), \( SDOC \) the concentration of recalcitrant DOC in the soil solution (mg kg\(^{-1}\)), \( k_{sc} \) the first order decomposition rate of SDOC (d\(^{-1}\)), and \( n \) is a parameter that determines the fraction of depolymerised compounds that flows into either the recalcitrant or the biodegradable DOC pool. This parameter \( n \) has a range of 0-1 (unit less) where a value of 1 indicates that all depolymerised compounds flow into BDOC and a value of 0 indicates that all depolymerised compounds flow into SDOC.

Bacterial cells require energy to maintain cellular activities and replace degraded proteins, even when not dividing. This maintenance energy is usually modelled by assuming that per unit mass maintenance is constant, resulting in:

\[
C_{\text{ma}} = C_m * K_m \tag{A6.9}
\]

where \( K_m \) is the maintenance energy (g g\(^{-1}\)).

Recalcitrant dissolved organic N

The change in recalcitrant DOC may also be controlled by sorption processes, depolymerization (breakdown to smaller organic compounds), and new inputs originating from soil microbial biomass, and old and fresh organic matter. Hence, the concentration SDOC can be described by:

\[
\frac{dC_{\text{SDOC}}}{dt} = -k_{sc} * C_{\text{SDOC}} - \frac{Q_{\text{max}C} * pC * C_{\text{SDOC}}}{1 + pC * C_{\text{SDOC}}} + b + C_{\text{dep}} * \frac{(1 - n)}{n} \tag{A6.10}
\]
General introduction

Soil and crop C

Soil organic C is subsequently calculated from the first order decomposition of the organic matter and the changes occurring to adsorption and sorption of BDOC and SDOC.

\[
\frac{dC_s}{dt} = -k_s \cdot C_s + \frac{Q_{maxC} \cdot pC \cdot C_{BDOC}}{1 + pC \cdot C_{BDOC}} + \frac{Q_{maxC} \cdot pC \cdot C_{SDOC}}{1 + pC \cdot C_{SDOC}} \quad \text{A6.11}
\]

\[
\frac{dC_p}{dt} = -k_p \cdot C_p \quad \text{A6.12}
\]

Nitrogen dynamics

The change in BDON and SDON are calculated similarly to BDOC and SDOC with parameters Y_N representing the N costs to build 1 unit microbial C, and Q_maxN and pN the Langmuir parameters describing adsorption kinetics of BDON to the soil matrix. The inputs of BDON from depolymerised organic matter are calculated assuming constant C-to-N ratio’s for biomass N (CN = 10), native soil N, and crop N. Since micro-organisms are also able to immobilize inorganic N when N limits C uptake, we assume that microbial uptake of C is not limited by N when sufficient inorganic N is present.

Mathematically, the microbial growth can be calculated as:

\[
u = u_{max} \cdot \left( \frac{C_{BDOC}}{K_{BDOC} + C_{BDOC}} \right) \cdot \max \left[ \frac{C_{BDON}}{K_{BDON} + C_{BDON}}, \frac{C_{NO3}}{K_{NO3} + C_{NO3}} \right] \quad \text{A6.13}
\]

where \( u \) is the actual microbial growth rate (d\(^{-1}\)), \( C_{NO3} \) the aqueous concentration of nitrate in the soil (mg kg\(^{-1}\)), and \( K_{NO3} \) the half-saturation constant for microbial growth on NO\(_3\) (mg kg\(^{-1}\)).

Microbial biomass N is calculated from microbial biomass C, assuming a constant CN ratio for the biomass.
The amount of NO$_3$ immobilized ($N_{im}$) can be calculated as the difference in N production for the situation that DON limits the microbial growth or not. Hence, net inorganic N mineralization can be calculated from:

$$\frac{dN_m}{dt} = \frac{dC_m}{dt} \times \frac{1}{CN_b}$$  \hspace{1cm} \text{A6.14}$$

Isotope tracing of added $^{15}$N from one pool to another can be calculated by multiplying the change of N with its enrichment (g $^{15}$N per g $^{14+15}$N):

$$\frac{d^{15}N}{dt} = \frac{dN}{dt} \times \frac{^{15}N_{source}}{N_{source}}$$  \hspace{1cm} \text{A6.16}$$

Where $dN/dt$ denotes the total N flow from one pool to another, and the $^{15}N_{source}/N_{source}$ represents the $^{15}$N enrichment of the N source.
Summary

Predicting the potential of soils to supply N is of considerable importance to maximize agricultural N use efficiency and to minimize environmental losses (Chapter 1). This research examines and evaluates the current soil testing approach, which uses extractable organic N (EON) fractions to predict soil N supply. In more detail, my objectives are:

- to evaluate all common chemical extraction methods for their ability to estimate the potential of soils to supply N (Chapters 3, 4);
- to quantify the influence of methodology, soil characteristics, environmental factors and nutrient management on the concentration of DON and EON in soils (Chapter 2);
- to investigate whether and how dissolved and extractable organic N fractions are involved in N mineralization using $^{15}$N tracing (Chapters 5, 6, 7);
- to evaluate the importance and applicability of the organic N fractions to improve N fertilizer strategies at the farm-scale (Chapter 8).

Evaluation of common chemical extraction methods

This study is the first one to quantitatively evaluate the overall predictive value of EON fractions using the meta-analysis approach, in which all chemical extraction methods developed during the last 100 years are included (Chapter 3). All tested EON fractions are positively related to the soils’ potential to supply N, and they explain on average 47% of the variation in soil N supply. Best predictions (averaged $R^2 > 57\%$) are obtained when EON is extracted with hot CaCl$_2$, acid KMnO$_4$, acid K$_2$Cr$_2$O$_7$, hot water or hot KCl. However, the majority of EON fractions perform either worse than or similarly to total N as a predictor of soil N supply. As expected, predictions of mineralizable N are significantly worse when mineralization is measured in the field compared with measurements under controlled conditions. In both situations, however, the uncertainty of the predicted soil N supply is still too big for serious improvement of fertilizer management.
I additionally applied multivariate statistical modelling to account for multi-collinearity among EON fractions, total N and soil properties in an incubation experiment with 98 Dutch agricultural soils (Chapter 4). This analysis shows that mineralization of N is primarily related to the size of organic N pools and fractions present. These organic N pools and fractions explain 79% of the variation in mineralizable N whereas other soil variables, particularly texture related variables, explain an additionally 8%. This multivariate analysis also shows that both total and extractable organic N reflect the same soil property, likely the soil organic matter content. As a consequence, i) the exact EON fraction used is less important, and ii) the combination of different EON fractions with or without total N does not provide extra information on the amount of mineralizable N compared to one single EON fraction. These observations are not limited to our incubation experiment: performing the same multivariate analysis on 39 published datasets results in similar observations (Ros et al., 2011e).

Factors that may control the concentration of DON and EON in soils

Chapter 2 examines the influence of methodological and environmental factors on the concentration of DON and EON in soils using a meta-analysis approach based on 127 studies. Dissolved and extractable N are neither similar in size nor similarly affected by the tested factors. The influence of factors affecting EON generally decrease in the order of methodology (10–2400%), followed by environment (11–270%) and management (16–77%). In contrast, DON concentrations are primarily influenced by management: different land use and fertilisation cause a variation of 37–118%. Methodological factors affecting DON have not been assessed due to limited data availability. The large range in EON as affected by different methodology emphasizes the importance of using standardized methods for the determination of EON.

Role of DON and EON in N mineralization

To further understand the role and functionality of organic N fractions in soil, I developed and tested a micro-diffusion method to analyse the \(^{15}\)N isotopic signa-
tture of organic N fractions in soil solutions and extracts (Chapter 5). Three off-
line techniques for measuring the $^{15}N$ signature of DON in presence of inorganic
N are tested.

In Chapter 6, this micro-diffusion method is used to test whether the source and dynamics of DON and CaCl$_2$ extractable organic N (either performed on oven dried or field-moist soil) differ upon soil amendment with $^{15}$N labeled ryegrass residue. The sampling procedure significantly affect the amount, but not the dynamics and origin of both organic N pools. They show all a significant increase upon crop amendment and return to their background concentrations within 10 to 30 days. The agreement in dynamics, $^{15}$N enrichment and C-to-N ratio’s suggest that dissolved and tested EON fractions have a similar role in N mineralisation.

Chapter 7 evaluates whether and how DON and EON are involved in N mineralization and how they interact with microbial biomass and the soil solids. Extractable organic N fractions are not only obtained with CaCl$_2$, but also with K$_2$SO$_4$, and hot water. The dynamics and isotopic signature of all aforementioned N fractions are studied during a 129-day incubation where $^{15}$N labeled radish residues have been applied. Residue application again releases a pulse of biodegradable and recalcitrant DON that temporarily dominates the soil DON and EON pools. The majority of DON and EON (> 80%) is derived from soil organic matter and is comprised of relatively recalcitrant compounds (turnover > 21 days). Their isotopic signature differs from microbial biomass and mineralized N likely due to fast (minutes to hours) decomposition and sorption. As a consequence, current sampling techniques collect a DON fraction that is comprised of (intermediate) decomposition waste products rather than of the most bio-available N compounds. The heterogeneous composition of DON limits the exact quantification of DON fractions, and additional simulation modelling is necessary to quantify the contribution of DON fractions to N mineralization.

**Application of soil testing in fertilizer management**

Finally, the results of the previous chapters are synthesized in Chapter 8 in order to evaluate the potential of soil tests to improve fertilizer N management.
Predicting soil N mineralization at farm-scale. All 20 EON fractions that have been tested significantly reflect the potential of soils to supply N and may have the potential to improve the prediction of soil N mineralization. However, the uncertainty of the predicted soil N supply is still too big for serious improvement of fertilizer management.

The fate of EON fractions in N mineralization, in particular those fractions that are obtained with weak hydrolyzing salt solutions, is comparable to that of dissolved organic N (DON). Both DON and EON can be considered as (intermediate) decomposition waste products that are in an abiotic and biotic controlled equilibrium with total N. Therefore, their relationship with soil N supply likely reflect that both DON, EON and soil N supply are mutually dependent on total N. An increase in total N is then associated with an increase in N mineralization and subsequently with an increase in decomposition waste products. Hence, the question ‘how are EON fractions involved in N mineralization’ can be changed in ‘how do both EON and soil N supply depend on total soil N’ in order to understand the relationship between EON and soil N supply.

The dependency of soil N supply on methodological and environmental issues strongly encourages more effort to be put into validation and up-scaling, particularly regarding the quantification of the differences between laboratory and field experiments. I advocate to adapt the current soil testing approach from a static soil testing procedure to a more dynamic approach, including simulation modelling of those environmental factors that control N mineralization. The exact EON fraction that can be used in such an approach is less important and practical considerations may be decisive to select one for routine application in soil analysis. In conclusion, a holistic approach, which considers spatial and temporal variability of both soil N supply and crop N demand, may provide a successful approach to improving fertilizer management at the farm-scale.
Samenvatting

Het voorspellen van stikstof (N) mineralisatie in de bodem kan een substantiële bijdrage leveren aan het verhogen van de N benutting in agrarische ecosyste- men. Eerder onderzoek heeft namelijk laten zien dat de productie van N via mineralisatie, ook wel N levering genoemd, een hoeveelheid stikstof kan produc- ceren variërend tussen 20 en 200 kg N ha⁻¹. Het kwantificeren van deze N leve- ring maakt het vervolgens mogelijk om het totale N aanbod (via bodem en be- mesting) te koppelen aan de N behoefte van het gewas. Hierdoor blijft een hoge productie en gewaskwaliteit gehandhaafd, en kan het mogelijk N verlies naar het milieu worden beperkt.

Dit onderzoek evalueert het gebruik van bodemtesten om een inschatting te maken van de N levering uit de bodem. Een bodemtest is een chemische ana- lyse van een bodemextract waarin een specifieke organische N fractie wordt ge- analyseerd. Deze specifieke fractie noem ik ‘extraheerbaar organisch stikstof’, afgekort als EON. Het onderzoek van de afgelopen 100 jaar laat zien dat de concentratie EON in een bodem vaak (lineair) samenhangt met de hoeveelheid N dat beschikbaar kan komen gedurende een groeiseizoen. Dit geeft aan dat deze bodemtesten gebruikt kunnen worden om een inschatting te geven van de N leve- vering. Geen van de ontwikkelde bodemtesten levert echter consistent een goede voorspelling van de N levering. De grote variatie tussen studies roept zelfs de vraag op of deze bodemtesten überhaupt in staat zijn om onder alle omstandig- heden een goede voorspelling te geven van de N nalevering. Overigens is tot op heden onduidelijk waarom deze relatie tussen een EON fractie en de N naleve- ring van een bodem bestaat. Is de extraheerbare N fractie de voornaamste ener- gie- en nutriënten bron voor bacteriën en schimmels? Wat is hierin de rol van opgelost organisch N (afgekort als DON), waarvan aangenomen wordt dat de productie ervan de bottleneck vormt in het gehele N mineralisatie proces?

De vragen die centraal staan in dit onderzoek zijn: (i) is een chemische bodemtest geschikt om de N levering van de bodem te voorspellen, en zo ja, wel- ke van de ontwikkelde testen levert het beste resultaat? (ii) wordt de concentra-
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tie EON voornamelijk bepaald door de extractiemethode, de bodemeigenschappen, het landgebruik, de bemestingsgeschiedenis of door weersinvloeden, en wat zegt dat over hun toepassing in bemestingsadviezen? (iii) zegt de hoeveelheid EON iets over de hoeveelheid N dat kan mineraliseren? Is er een causaal verband tussen beide variabelen? en (iv) kunnen deze EON fracties gebruikt worden om N mineralisatie te voorspellen, en zo ja, onder welke condities is dat wel of niet mogelijk?

Evaluatie bestaande bodemtesten

Deze studie kwantificeert de voorspellende waarde van alle gebruikte bodemtesten die ontwikkeld zijn gedurende de afgelopen 100 jaar met behulp van een statistische techniek genoemd meta-analyse (Hoofdstuk 3). Met deze techniek is het mogelijk om een gemiddelde voorspellende waarde (gebaseerd op alle gepubliceerde experimenten) voor elke bodemtest te berekenen: elk experiment of studie krijgt een wegingsfactor die gekoppeld is aan de kwaliteit van de desbetreffende studie. Uit deze meta-analyse blijkt dat alle bodemtesten positief gerealiseerd zijn aan de N levering van een bodem: een EON fractie verklaart gemiddeld 47% van de variatie in de N levering. Het merendeel van deze bodemtesten geeft echter geen beter resultaat dan een voorspelling gebaseerd op de totale hoeveelheid N in een bodem. Statistisch gezien zijn EON fracties daarom niet a priori te prefereren boven de totale hoeveelheid N om een goede voorspelling van de N levering te geven. Zoals verwacht is de voorspelling van de N levering beter wanneer deze wordt gemeten onder gecontroleerde omstandigheden (constant vocht en temperatuur). Ondanks dit verschil is de onnauwkeurigheid van en onzekerheid rond de voorspelde N nalevering in het veld en in het laboratorium nog te groot om enige substantiële verbetering aan te brengen in N bemestingsadviezen.

Aanvullend op deze meta-analyse heb ik gebruik gemaakt van een multivariabele statistische analyse, omdat ik rekening wilde houden met het feit dat veel EON fracties sterk aan elkaar gerealiseerd zijn (Hoofdstuk 4). Deze EON fracties zijn daarnaast ook sterk gekoppeld aan de totale hoeveelheid N in de bodem. In mijn incubatie experiment met 98 agrarische bodems verklaarden de
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totale hoeveelheid $N$ en de EON fracties ongeveer 79% van de variatie in $N$ nalevering. Dit suggereert dat EON fracties de totale hoeveelheid organische stof reflecteren, en de keuze voor een specifieke EON fractie is daarmee niet doorslaggevend voor een goede voorspelling van de $N$ nalevering. Het betekent ook dat het combineren van verschillende EON fracties (of bodemtesten) niet tot een betere voorspelling zal leiden. Andere bodemeigenschappen zoals de kwaliteit van de organische stof en textuur gerelateerde variabelen kunnen in aanvulling op de EON fracties maximaal 8% van de variatie in $N$ nalevering verklaren. Een gedifferentieerd $N$ bemestingsadvies voor verschillende textuurgroepen zou daardoor een meerwaarde kunnen opleveren boven een uniform $N$ advies.

De bovenstaande observaties zijn niet alleen geldig in dit incubatie experiment, maar worden ondersteund door mijn analyse van 39 andere (gepubliceerde) datasets (Ros et al., 2011e).

Factoren die de concentratie opgelost en extraheerbaar $N$ beïnvloeden

In hoofdstuk 2 onderzoek ik de invloed die methodologie, bodemeigenschappen en omgevingsfactoren hebben op de concentratie opgelost en extraheerbaar $N$ in de bodem. Ik gebruik hiervoor opnieuw de meta-analyse benadering. Uit deze analyse blijkt dat de opgeloste en extraheerbare $N$ fracties niet alleen verschillen in grootte en samenstelling, maar ze worden ook op een verschillende manier beïnvloed door de onderzochte factoren. Factoren die een groot effect hebben op de concentratie EON zijn methodologie (effect varieert tussen 10 en 2400%), weersomstandigheden (effect variërend tussen 11 en 270%), en nutriënt management en landgebruik (effect varieert tussen 16 en 77%). De concentratie opgelost organisch $N$ daarentegen wordt voornamelijk beïnvloed door management: landgebruik en nutriënt management veroorzaken een variatie in DON variërend van 37 tot 118%. Helaas kon het effect van methodologie op de concentratie DON niet worden gekwantificeerd door een gebrek aan data.

Rol van opgelost en extraheerbaar $N$ in $N$ mineralisatie

Om de rol en functie van organische $N$ fracties in de bodem verder te onderzoeken heb ik een microdiffusie methode ontwikkeld en getest waardoor het mo-
gelijk is om de (stabiele) isotopen samenstelling van deze fracties te analyseren (Hoofdstuk 5). Ik testte drie verschillende protocollen om het $^{15}$N ‘signaal’ van opgelost organisch N nauwkeurig te meten.

In hoofdstuk 6 is deze methode gebruikt om te onderzoeken of het gedrag en de samenstelling van opgelost en extraheerbaar organisch N verschillen na het toedienen van $^{15}$N gelabelde gewasresten. De EON fracties zijn gemeten in een CaCl$_2$ extract van een gedroogde en een veldvochtige grond. De opgeloste organisch N fractie is bepaald in een afgecentrifugeerd bodemvocht monster. Grond extractie resulteerde in een significante toename van de hoeveelheid organisch N in het extract in vergelijking met de hoeveelheid opgelost organisch N in de bodemoplossing, met name na het drogen van de grond. Ondanks dit verschil in concentratie, was er geen verschil in het $^{15}$N signaal, de temporele variatie, en in de C-N verhouding van de drie organische N fracties. De concentratie organisch N in de bodemoplossing en in de beide extracten nam sterk toe na het inwerken van gewasresten, maar keerde terug naar de uitgangssituatie binnen 10 tot 30 dagen. De overeenstemming in samenstelling en gedrag suggereert dat deze fracties eenzelfde rol vervullen in N mineralisatie.

Hoofdstuk 7 gaat dieper in op deze rol van opgelost en extraheerbaar N in het mineralisatie proces. Aanvullend wordt onderzocht hoe deze fracties beïnvloed worden door de microbiële biomassa en de minerale delen in de bodem (adsorptie en desorptie processen). Het gedrag en $^{15}$N signaal van opgelost organisch N en drie EON fracties werd gevolgd over een 129 dagen durend incubatie experiment waaraan gelabeld bladrammas was toegevoegd. Uit dit experiment blijkt dat de toevoeging van gewasresten een puls aan organische moleculen doet vrijkomen die tijdelijk de hoeveelheid en samenstelling van de opgeloste N fractie bepalen. Na enkele dagen is de samenstelling van de organische N fractie weer gelijk aan de uitgangssituatie waarbij het merendeel bestaat uit relatief moeilijk afbreekbare componenten (niet afbreekbaar binnen 21 dagen). Het $^{15}$N signaal van de organische N fracties verschilde van dat van de microbiële biomassa en de geproduceerde NO$_3$, waarschijnlijk door een snelle (op een tijdschaal variërend van minuten tot uren) mineralisatie en adsorptie van gelabeld N. Als een consequentie hiervan bestaat het merendeel van de opgeloste en
extraheerbare N uit relatief moeilijk afbreekbare afbraakproducten. Alhoewel deze fracties nog steeds een bijdrage leveren aan de geproduceerde NO₃, toch zijn deze fracties niet de meest biologisch beschikbare N vorm in de bodemoplosing. De heterogene samenstelling van de opgeloste N fracties bemoeilijkt het nauwkeurig inschatten van de mineralisatie snelheid van deze fracties, en aanvullende simulatie modellering is nodig om deze bijdrage alsnog te berekenen. De resultaten van deze studie maken in ieder geval duidelijk dat er geen positief en causaal verband bestaat tussen de totale concentratie organisch N in een bodemextract en de gemiddelde mineralisatie snelheid van de individuele moleculen in de desbetreffende fractie.

**Gebruik van EON fracties in nutriënt management**

De resultaten van voorgaande hoofdstukken zijn geïntegreerd in een afsluitend discussie hoofdstuk (hoofdstuk 8). Uit mijn onderzoek blijkt dat alle ontwikkelde bodemtesten de potentie hebben om de N nalevering van een bodem te schatten. Echter, de huidige onzekerheid op de voorspelde N levering is helaas nog te groot om een substantiële verbetering aan te brengen in bemestingsadviezen.

De rol die EON fracties spelen in N mineralisatie, in het bijzonder die fracties die geëxtraheerd kunnen worden met zwakke zoutoplossingen (zoals CaCl₂ of K₂SO₄), is vergelijkbaar met de rol van opgelost organisch N. Beide N fracties kunnen worden beschouwd als afbraakproducten die vrijkomen gedurende het mineralisatie proces. De concentratie van deze N fracties in de bodem is in evenwicht met de totale hoeveelheid aanwezig N, en wordt beïnvloed door chemische en biologische processen. De positieve relatie tussen de EON fracties en de N nalevering reflecteert daarom de samenbindende rol van bodem organisch stof. Een hoger percentage organische stof in een bodem resulteert in een hogere mineralisatie snelheid, in een hogere concentratie afbraakproducten, en tegelijkertijd in een hogere N nalevering. Als het verband tussen organische N fracties en de N nalevering samenhangt met de hoeveelheid organische stof, dan kan de vraag ‘welke rol spelen EON fracties in N mineralisatie?’ vervangen worden door de vraag ‘hoe hangt de hoeveelheid EON en de N nalevering af van de hoeveelheid organisch stof in de bodem?’ Het betekent ook dat bodemtesten al-
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leen toepasbaar zijn wanneer het bodemsysteem in een relatief evenwicht verkeert: een verstoring van de evenwichtsrelatie tussen EON en totaal N verstoort de relatie tussen EON en N levering. Dit betekent concreet dat het onmogelijk is om de mineralisatie van een bodem te voorspellen via een bodemtest als deze recent is bemest met dierlijke mest of gewasresten.

De invloed van methodologie en weersomstandigheden op de actuele N nalevering benadrukt het belang van een goede vertaalslag van een laboratorium experiment naar de situatie in het veld. De huidige ‘statische’ bodemtest benadering zal daarom richting een ‘dynamische’ benadering moeten ontwikkelen om tot een goede voorspelling van de N levering in het veld te komen. Deze meer holistische benadering, waarbij rekening wordt gehouden met de ruimtelijke en temporele variatie in N nalevering en gewas vraag, zal mijns inziens tot een succesvolle aanpak leiden om het N management op bedrijfsniveau te verberen. Inzicht in het mineralisatie proces en het kwantificeren van de effecten van organische stof kwaliteit, bodemstructuur en weersomstandigheden is daarbij essentieel.
Curriculum vitae

Gerard H. Ros was born in Zwolle, the Netherlands, on 19 August 1980. He completed his secondary (VWO) in 1998, and in the same year, he started his study ‘Soil, Water and Atmosphere’ at Wageningen University. He specialized in both ‘Soil Science and Plant Nutrition’ and ‘Geohydrology’. For his first MSc thesis, he studied the accuracy of simple simulation models and a biological incubation assay to predict nitrogen (N) mineralization in an arable field. His second MSc thesis was a pilot study investigating the possibility of gravimetric detection of water storage changes in unsaturated soil. To finalize his studies, he did his internship at Nutrient Management Institute where he did research on the role of dissolved organic matter in soils, agricultural management strategies to improve the N use efficiency, the potential of organic waste composting to improve soil fertility in developing countries, on management strategies to reduce the emission of agrochemicals in Dutch agriculture, and he also developed an adapted N fertilizer recommendation for corn. In January 2006, he started as junior specialist in geo-hydrology at Witteveen+Bos, with a special focus on the quantification of nutrient flows. In January 2007, he started his PhD research in the Soil Quality Group at Wageningen University.
List of publications and presentations

Peer reviewed papers


Submitted/ in preparation

Presentations at international scientific meetings
Meta-analysis of soil data illustrated by an analysis of factors controlling DOM. Soil & Water, 9-10 June 2008, Zeist, the Netherlands.

Soil tests predicting N mineralization: a statistical evaluation of their predictive value. 16th N workshop ‘Connecting different scales of nitrogen use in agriculture’, 28 June - 1 July 2009, Turin, Italy.

A functional evaluation of four in situ collected or extracted DOM pools. EGU annual congress, 2-7 May 2010, Vienna, Austria.

Dynamics of dissolved organic N in soil investigated by $^{15}$N isotope tracing. Organic matter stabilization and ecosystem functioning, 19-23 September 2010, Presqu’ile de Giens, France.


Predicting soil N mineralization; relevance of extractable organic matter fractions. EGU annual congress, 3-8 April 2011, Vienna, Austria.


Presentations at national (scientific) meetings

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Dissolved organic Nitrogen: nut voor boer en samenleving. STW Annual Congress, 8 October 2009, Utrecht, the Netherlands

Is there a future for soil nitrogen testing? Bodembreed, 2-3 December 2010, Lunteren, the Netherlands
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Additional info

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CERTIFICATE

The Netherlands Research School for the Socio-Economic and Natural Sciences of the Environment (SENSE), declares that

Gerardus Hendrik Ros

born on 19 August 1980 in Zwolle, The Netherlands

has successfully fulfilled all requirements of the Educational Programme of SENSE.

Wageningen, 17 June 2011

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- Site-specific training, Spectraelectron DGM, EION
- Review and meta-analysis of the predictive value of soil tests
- Review of the factors controlling dissolved and extractable organic N
- Supervisor, practical, BSc course 'Biological Interactions in soils'
- Supervisor, BSc course 'Introduction Environmental Science'
- Supervisor, MSc course 'Academic Consultancy Training'

Oral and Poster Presentations
- Meta-analysis of soil data: illustrated by an analysis of factors controlling DON and DOC, Soil & Water, 9 – 10 June 2008, Zinat, The Netherlands
- Predicting N mineralization – a statistical evaluation of the predictive value of soil tests, Soil Organic Matters, 23 – 25 June 2009, Rothamsted, UK
- Dynamics of dissolved organic N in soil investigated by 15N isotope tracing, Organic matter stabilization and ecosystem functioning, 13 – 23 September 2010, Frascati de Gignes, France
- Isotopic Analysis of Dissolved Organic Nitrogen in soils, SIF symposium From Sources to Success: Contributions of Light Stable Isotopes to Environmental Sciences, 15 – 16 December 2010, Davis, USA
- Predicting soil N mineralization: relevance of extractable organic matter fractions, European Geosciences Union annual congress, 3 – 8 April 2011, Vienna, Austria

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