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Immobilization and mineralization of nitrogen in pasture soil

Samenvatting:
Immobilisatie en mineralisatie van stikstof in grasland

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Abstract

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The results obtained from turf samples indicate that growing plants are mainly responsible for the accumulation of soil organic nitrogenous compounds. Mixing of the soil of turf samples containing living plants did not stimulate the release of soil organic N.

Addition of unlabeled $(NH_4)_2SO_4$ did not promote the liberation of labeled nitrogen (N^{15}) recently immobilized in turf samples with living grass plants. This labeled part was mineralized more readily than the originally present soil organic matter upon killing of the grass plants.

The amino acid patterns of the hydrolysates of pasture soil, arable land and the humic acids of these soils were rather similar, resembling the amino acid composition of the hydrolysates of the 'humic acids' produced by streptomycetes in a glycerol-nitrate medium.

Soil organic matter was used as the only N source for the growth of a proteolytic *Pseudomonas* strain. The results obtained suggest that more protein-like material is incorporated in the soil organic matter of pasture than in that of arable land. The availability of N 'humic acids', synthesized by a *Streptomyces* strain, to the *Pseudomonas* sp. was similar to that of humic acids extracted from grassland by NaOH.

The author graduated on November 3th, 1972 as Doctor in de Landbouwwetenschappen at the Agricultural University, Wageningen, the Netherlands, on a thesis with the same title.

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Contents

1 Introduction

It is a well-known fact that the amount of organic nitrogen in grassland soil tends to increase with time. This accumulation of organic nitrogen continues for many years ('t Hart, 1950)[1]. An equilibrium is reached after several decades when the production of soil organic nitrogen is equal to its decomposition. In soil of arable land the amount of organic nitrogen generally remains constant or decreases.

Clement & Williams (1967) showed that organic nitrogen accumulated in the top 15 cm of soil under grass at an annual rate of 0.005 % of air-dried soil. When the weight of the soil layer amounts to 2.5×10^6 kg of dry soil, this percentage corresponds with an annual increment of 125 kg nitrogen per hectare over a depth of 15 cm. This phenomenon partly explains the fact that the recovery of fertilizer nitrogen in the shoots of the plants of grassland is often lower than 60 % (Mulder, 1949; van Burg 1962, 1970).

In the present investigation, an attempt has been made to study the mechanism responsible for the accumulation of organic nitrogen in grassland soil.

Pot experiments with soil of arable land and with turf samples of grassland have been performed. As the organic nitrogen content of soils generally is so high that small changes cannot be determined by conventional methods, labeled fertilizer nitrogen has been used. The effect of the presence of living plants and the effect of disturbing and mixing the turf samples on nitrogen transformations in the soil have been studied. For this reason balance-sheets of soil nitrogen and labeled fertilizer nitrogen have been determined.

Ploughing up of grassland is followed by a rapid decline of the soil organic matter content ('t Hart, 1950). It is likely that the chemical composition of soil organic matter of grassland differs from that of soil of arable land. This has been checked by estimating the amino acid pattern of hydrolysates of both types of soil. From the literature it is known that streptomycetes are able to synthesize humic acid-like compounds and that these organisms constitute a greater part of total cell count in soil of grassland than in soil of arable land. For these reasons the production of *Streptomyces* 'humic acid' has been investigated. The amino acid composition of the hydrolysed humic acid-like compounds has been compared with that of humic acids isolated from soil of arable land and grassland, respectively.

By analytical procedures used in soil chemistry it is impossible to determine the

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[1] For references Chapter 1: see Section 2.5, page 13.

fraction of soil organic matter which becomes easily decomposable after ploughing up of grassland. In the present study an attempt has been made to obtain more quantitative information about this fraction by determining the growth of a proteolytic *Pseudomonas* strain in a medium containing soil organic matter as the only nitrogen source.

2 Review of literature on the accumulation of soil organic nitrogen in pastures and on the biosynthesis of humic acid-like substances

2.1 The fate of fertilizer nitrogen in grassland soils

Many investigations have shown that the recovery of fertilizer nitrogen in the shoots of grass plants of permanent pastures is low. Mulder (1949), Cunningham & Cooke (1958), van Burg (1962, 1970) and Woldendorp (1963) recorded that this recovery seldom exceeds 60 %, it usually being less than 50 %.

Losses by leaching of fertilizer nitrogen from grassland were recorded by Woldendorp et al. (1966). In lysimeters filled with sandy soil and sown with grass, scarcely any losses were found to occur when ammonium nitrate was applied during the growth season, except when heavy rainfall followed application. Therefore, losses of available fertilizer nitrogen in grassland soil must be due to volatilization or to incorporation in the roots or in soil organic matter. Woldendorp (1963) determined nitrogen balance-sheets in turf samples of permanent grassland using labeled fertilizer nitrogen. Under varying experimental conditions where leaching was impossible, 45–65 % of a nitrate dressing was found in the shoots, 10–25 % in the roots, and 7–22 % in the soil, while 10–40 % had been lost by denitrification. When ammonium nitrogen had been supplied, about 10 % was lost by denitrification after its transformation to nitrate by nitrifying bacteria.

Grassland soils are characterized by the rapid disappearance of fertilizer nitrogen when applied during the growth season. Richardson (1938) observed that the added fertilizer was entirely absorbed within at most a fortnight. Without added fertilizer, the inorganic nitrogen content of grassland soil is low throughout the whole year. Richardson found values for ammonium nitrogen between 2 and 8 and for nitrate nitrogen between zero and 2 mg per kg dry soil. The low inorganic nitrogen content of soil of permanent pasture has most commonly been attributed to a high nitrogen absorption rate due to microorganisms which are stimulated by the large quantities of organic materials added to the soil by the grass roots. A different explanation has been given by Theron (1951, 1963). He found that a very considerable mineralization of nitrogen took place as soon as the grass cover was removed, though large quantities of root residues still remained in the soil. He suggested, therefore, an inhibition of the mineralization, due to a toxic effect of an exudate of the living grass roots on the bacteria mineralizing nitrogen.

2.2 Accumulation of soil organic nitrogen in grassland

It is a well-established fact that large increases in organic nitrogen occur in grassland soil. Richardson (1938) estimated that the soil organic nitrogen can increase for more than 150 years. After such a long period of time, the production and decomposition of soil organic matter will be equal and the content of soil organic nitrogen will remain constant. Determinations of soil nitrogen changes under pure grass swards (Parker, 1957; Clarke, 1970) showed annual increases of soil nitrogen of the order of 20 to 40 kg per hectare, although no fertilizer nitrogen was applied. Recent investigations of Barrow (1969) showed an annual increase of 38 kg N per hectare in the top 12.5 cm of sandy soils under pastures, which were only fertilized with superphosphate. The accumulated nitrogen in these pastures was mainly derived from symbiontic nitrogen fixation due to the presence of clover. Clement & Williams (1967) analysed all the organic materials – roots and other underground plant organs were included in the soil samples – and observed that under a ryegrass/white clover sward, organic nitrogen accumulated in the top 15 cm at an annual rate of 100 to 110 kg N per hectare. Application of 45 kg fertilizer nitrogen per hectare resulted in an annual increment of 125 kg N per hectare. With an annual dressing of 314 kg N, increases under grazed leys averaged up to 180 kg N per year.

The mechanism of nitrogen accumulation under grass is imperfectly understood. In the presence of living plants, carbonaceous material is added to the soil by dead root hairs, dead root cells and root excretions. These plant-derived compounds may be used by microorganisms as energy source and as carbon source to build up their cell material. The formation of microbial cells includes immobilization of nitrogen in their cell organic matter. This immobilized nitrogen may be derived from nitrogenous fertilizers and from mineralization of soil organic nitrogen, of nitrogenous plant materials and of microbial cells. In grassland, the production of carbonaceous material may be so high that more nitrogen is immobilized than mineralized, resulting in accumulation of soil organic nitrogen.

The soil organic matter is stable as long as the soil is under grass. Ploughing up of grassland soils is followed by a rapid decline of the organic matter content ('t Hart, 1950). Due to this treatment, the plants are killed and the environmental conditions in the soil are changed. Killing the grass plants by ploughing will arrest the continuous flow of root-derived carbonaceous material resulting in a decrease of soil organic nitrogen and an increase of inorganic nitrogen. In the following, the changes caused in the soil of grassland by ploughing will be discussed in order to clarify the circumstances which are leading to accumulation of soil organic nitrogen in grassland.

2.2.1 Influence of living grass plants on the supply of carbonaceous material to the soil

It is rather difficult to determine the amount of carbonaceous material which is delivered by living plants to the soil. Goedewaagen & Schuurman (1950a) estimated that the annual production of roots, produced by grass crops, amounts to approximately 5000 kg per hectare. According to Shamoot et al. (1968) the quantities of organic debris (excluding roots) remaining in the soil per 100 units of root weight ranged between 20 and 50 units. For grassland these figures correspond with an annual production of 1000 to 2500 kg per hectare. Due to the fact that part of these organic debris has already been decomposed to CO_2 during the experimental period, the total production will be even higher.

Goring & Clark (1949) showed that the mineralization of nitrogen was depressed by the presence of crops in comparison with the fallow soil. They ascribed this to the re-immobilization of mineralized nitrogen, caused by the microbial decomposition of root compounds with a low nitrogen content.

2.2.2 Influence of living grass plants on the moisture content of the soil

The accumulation of soil organic nitrogen in grassland may be caused by the continuous production of carbonaceous material by plants, but according to Harmsen & van Schreven (1955) other factors may also be responsible. They wrote, 'the accumulation of humus in grassland cannot entirely be explained by the generally higher amounts of plant residues produced in grassland and forest than in arable land, because even the scantiest vegetation on grasslands, prairies, forests, etc., is proved to be able to build up a certain level of humus, while even luxuriously growing crops with high applications of farm manure, compost, or green manure can never entirely avert the depletion of humus and total N in arable soil'. Harmsen (1951) applies the theory of Enders (1942) on humus formation to the situation occurring in grassland. This theory is based on an accelerated synthesis of humus by microbes under unfavourable conditions. These conditions, i.e., frequent and abrupt changes in humidity, temperature and irradiation, occur in the extreme surface of the soil and Harmsen (1951) therefore proposed to regard this soil layer as the main site of humus formation. The accumulated humus is expected to be transported to lower layers by biological activity.

The major part of the root system of grassland soils is concentrated in the surface soil. Goedewaagen & Schuurman (1950b) showed that 62 per cent of the total root mass was generally present in the top layer 0–5 cm and 87 per cent in the layer 0–20 cm This may have very important consequences as far as the moisture content of the surface soil of grassland is concerned. The grass plants will take up their water mainly from the layer 0–20 cm, causing a rapid decrease of the moisture content of the soil during periods of the growth season without rainfall. Although the fluctuation of the moisture content in the layer 0–20 cm may be less frequent than in the extreme surface of the soil, the theory of Enders about humus formation may also be valid for this deeper soil layer.

In contrast to the suggestions of Harmsen about humus synthesis, the experiments of Birch (1958) showed that intermittent drying and wetting of soil during incubation enhanced the amount of mineralized nitrogen. With plant residues added, it appeared that the more frequently the soil went through the drying and remoistening cycle the greater was the amount of mineralized plant nitrogen (Birch, 1964). The results of these experiments suggest that accumulation of soil organic nitrogen would be prevented by cyclic drying and wetting of the soil. However, this suggestion is not in agreement with the observed accumulation of organic matter in grassland soils. Two reasons for this deviation may be given.

1. In the soil samples without added plant residues tested by Birch (1958), carbonaceous material apparently did not come available at a sufficient rate to re-immobilize the mineralized nitrogen and to bring about accumulation of humus. The latter process can only occur when carbon and nitrogen are supplied by other sources than the original humus stock. In grassland the supply of available carbonaceous material may be so large that the mineralized nitrogen – eventually appearing in the soil after remoistening by rainfall following a dry period – is readily incorporated in proliferating microbial cells, as far as this nitrogen is not taken up by the grass plants. When the conditions change and become unfavourable for microbial growth (dry soil), humus formation might be favoured by the death of microbial cells.
2. In the soil samples containing plant residues tested by Birch (1964), it is possible that besides nitrogen mineralization of plant residues, the synthesis of humus had also been favoured by intermittent drying and wetting during the incubation period.

When it does not rain for a long period of time, the soil may become so dry that microbial decomposition of humus compounds and of plant residues will stop. The mineralization of soil organic matter increases with increasing moisture content, it being optimal at 50–60 % of the waterholding capacity (Jansson, 1958). Experiments of Grootenhuis (1961) indicate that more nitrogen is mineralized in ploughed pasture soils during a wet summer than during a dry summer. Robinson (1957) noted that at moisture levels between the wilting point and field capacity, the percentage mineralization increased with increasing moisture content. A small decrease in the moisture content below the wilting point was sufficient to prevent the substantial formation of mineral nitrogen. No mineralization occurred in air-dry soil.

In summary, it may be stated that the course of the moisture content in pasture is an important factor in the accumulation of soil organic nitrogen. Frequent changes of the moisture content may stimulate humus synthesis. A prolonged period of dryness may retard mineralization. This effect of moisture content on humus metabolism is expected to be less important in arable land where the plant roots may penetrate in deeper soil layers to absorb water. Moreover, this land is occupied by the crop only during part of the year, resulting in a more constant and higher level of the moisture content of the soil.

2.2.3 *Influence of disturbing the soil*

Woldendorp (1963) found that the mineralization rate in turf samples or permanent grassland with killed root systems was promoted by mixing the soil. An explanation of this effect may be that the poorly aerated soil aggregates are destroyed so that oxygen may penetrate more readily in all parts of the soil.

2.2.4 Influence of aeration

The observation of Woldendorp (1963) that denitrification is stimulated by the presence of living plants indicates that the oxygen concentration is grassland soil is low. The level of oxygen concentration which reduces nitrogen mineralization is not known. Experiments of Greenwood (1961) indicate that the respiration rates of simple organic compounds in water-saturated crumbs (mean radius 1.55 mm) of a loam soil were only slightly affected by reduction of the oxygen partial pressure in the atmosphere from 15 to 1.7 cm of mercury. Due to the inability of detecting any products of anaerobic decomposition at an oxygen partial pressure at which more than 80 % of the soil provided conditions for anaerobic decomposition, Greenwood suggested that these products diffuse into regions where sufficient oxygen is available to maintain aerobic metabolism. Novák (1971) investigated mineralization and humification under aerobic and anaerobic conditions. Greater amounts of humic substances were formed from straw under aerobic conditions. The products of anaerobic metabolism of straw and glucose were more readily mineralized and humified by the aerobic soil microflora than glucose.

These results may indicate that in grassland soils both the rate of mineralization and the rate of humification are stimulated by the occurrence of aerobic and anaerobic regions at the same time. It is likely that the level of oxygen concentration is dependent on the moisture content of the soil. As the moisture content of grassland is frequently changed, the same may be valid for the oxygen concentration.

2.3 Building units of nitrogenous soil organic matter

A large number of compounds of plant, animal, and microbial origin in varying stages of decay may be involved in humus production. Due to the fact that lignin is relatively resistent to biological attack, Wacksman & Iyer (1932) postulated that humic acids are formed by reaction between lignin and proteinaceous material in soils. Isolation of a small amount of a lignin-protein complex has been reported by Tinsley & Zin (1954) and by Jenkinson & Tinsley (1960). Mattson & Koutler-Andersson (1943) suggested that part of the soil nitrogen is present as complexes of oxidized lignin and ammonia. Studies by Sørensen (1962) showed that reactions between oxidized lignins and amino acids may occur during the biological decomposition of plant materials in soils.

However, there is no evidence that lignin accounts for a significant proportion of the organic matter of the soil. Although lignin yields appreciate amounts of phenolic aldehydes when oxidized with nitrobenzene in alkaline solution, only trace amounts are obtained from humic acids of soil organic matter (Bremner, 1955a; Morrison, 1958, 1963).

Hydrolysis of soils (Keeney & Bremner, 1964) and of humic acid preparations with 6 N HCl (Bremner, 1955b) showed that from 20–50 % of the total nitrogen in most soils and in humic acids is in the form of bound amino acids, and from 5–10 % is in the form of combined hexosamines. Ammonium nitrogen liberated by hydrolysis was found to account for 15–25 % of the total soil organic nitrogen.

There are several theories as to how the amino acids of soil organic matter are combined. Polymer formation of amino compounds (amino acids, peptides and proteins) with carbohydrates or their decomposition products (sugar, methyl glyoxal, etc.) has been suggested to occur by the so-called Maillard reaction (Maillard, 1912, 1917; Enders, 1942; Schuffelen & Bolt, 1950).

Swaby & Ladd (1962) proposed that amino acids are incorporated as single units during the oxidative polymerization of phenols. It is unlikely, however, that amino acids occur only as separate units, because Sowden (1966a, 1966b) showed the presence of peptides in hydrolysates upon partial hydrolysis of soil with concentrated acid at room temperature. Simonart et al. (1967) isolated a protein fraction from soil humic acid. Evidence for its occurrence in soil humic acids has also been obtained by showing that the proteolytic enzyme pronase releases α-amino acids from humic acids (Ladd & Brisbane, 1967).

Kononova & Aleksandrova (1959) suggested that organic residues of plant and animal origin are first decomposed to simpler compounds through the activity of microorganisms. Some of the relatively simple products may be utilized by microorganisms to synthesize humic substances.

Some authors (Laatsch et al., 1952; Flaig, 1950, 1964) suppose the occurrence of a reaction of ammonia, amino acids, peptides or proteins with oxidized phenolic compounds derived from lignin degradation or from microbial metabolism.

Much information about substances involved in humus formation has been obtained from studies with artificial humic acids. Haider et al. (1965) showed that in the presence of phenol oxidases only such phenols which have no methylated hydroxyl groups reacted with amino acid compounds. The polymeric reaction products of oxidized phenols with amino acids were stable against hydrolysis. It was also found that after hydrolysis only the N-terminal amino acid of incorporated peptides, which is bound to oxidized phenols, could not be recovered. Using serum albumin, it was found that there is a reaction of the N-terminal amino acid and also of the ε-amino group of lysine residues with phenols during oxidation. Ladd & Butler (1966) prepared phenolic polymers, either nitrogen-free or incorporating amino acids, peptides, or proteins, from *p*-benzoquinone and catechol under mild oxidative conditions. Results showed that polymers, in which peptides and proteins are incorporated, resemble humic acids more closely than those incorporating separate amino acids. Experiments with polymers incorporating peptides showed that the bond between the carbon atom of an aromatic ring and the nitrogen atom of an α-amino acid is far more stable to acid hydrolysis than peptide bonds.

2.4 Formation of humic acid-like substances by pure cultures of microorganisms

Under laboratory conditions pure cultures can give insight into the processes taking place in the natural environment. Many experiments about humus formation by pure cultures of fungi and actinomycetes have been described in the literature. An advantage of such model experiments is that the influence of different carbon sources (such as lignin, phenols and non-aromatic compounds) and different nitrogen sources (such as inorganic nitrogen, amino acids, peptides and proteins) on humic acid formation can easily be investigated.

Wieringa (1958) and Woldendorp (1963) pointed out that actinomycetes constitute a greater part of the total cell count in grassland than in non-grassland soil. Flaig & Kutzner (1960) found the number of peptone-browning streptomycetes isolated from grassland to be generally higher than that from arable land of the same soil type. The formation of humic acid-like compounds by cultures of such streptomycetes was shown by Scheffer et al. (1950), Laatsch et al. (1950), Flaig et al. (1952). Glycerol was generally used as the carbon source and amino acids or peptone as the nitrogen source. Von Plotho (1950) found a fall of pH of the nutrient medium after inoculation with actinomycetes, which was followed by a rise of pH when the culture solution darkened, indicating autolysis of the cells. No humic acids were formed below a pH value of 7.5. Küster (1952) concluded from his experiments with streptomycetes that the aromatic building units of humic acids are not only supplied by plants (lignin), but may also be derived from the metabolic products of microorganisms. Küster (1958) demonstrated that the peptone-browning reaction depends on the presence and the activity of phenol oxidases. Two types of these oxidases can be distinguished, viz. tyrosinase (catechol oxidase) oxidizing monophenols and *o*-dephenols, and laccase (*p*-diphenol oxidase) oxidizing *o*- and *p*-diphenols (Küster, 1955; Kutzner, 1968). When the phenols have been oxidized, they can polymerize, incorporating not only phenols but also nitrogenous substances. Matschke (1970) showed that many types of nitrogenous substances may be involved in these condensation reactions. He found 15 different amino acids in hydrolysates of humic acids isolated from cultures of *Streptomyces aureus*.

Kang & Felbeck (1965) extracted humic acid-like substances from spores and mycelium of *Aspergillus niger* with alkali. Elementary analysis, methoxyl content and other characteristics showed that the 'humic acids' isolated from spores more closely resembled soil humic acids than those isolated from the mycelium. The yields of humic acid-like compounds, expressed as percentage of ash-free tissue weight, amounted to 24 and 16 % respectively, for spores and mycelium.

Martin et al. (1967) isolated humic acid-like substances from *Epicoccum nigrum*, grown in a glucose-asparagine medium. The yield amounted to about 10 % of the organic material synthesized by this fungus. The authors concluded that certain microbes, even when provided with relatively simple organic carbon and nitrogen sources, are able to produce substances which are similar to naturally occurring humic materials. Addition of certain phenols increased yields of 'humic acid' by 70–200 %.

Haider & Martin (1967) isolated two phenols from the nutrient solution of *E.nigrum* during the first week of growth of this organism. It appeared that during later stages of growth the fungus altered these phenols by introduction of additional OH groups, by decarboxylation and by oxidation of methyl to carboxyl groups, forming a large number of different polyphenols. The synthesis of humic acid-like compounds coincided with the disappearance of the phenols, increase of ammonia concentration and rise of pH of the culture medium. The results obtained with C^{14}-labeled phenols indicate that most of the side chain and methoxyl carbon atoms were released as CO_2 or were utilized for cell synthesis, while most of the ring carbon was incorporated in 'humic acid', or remained in solution. Phenolases were not found in the nutrient solution of *E.nigrum*; a weak phenolase activity was detected in the mycelium. However, polyphenols identified in the nutrient solution before 'humic acid' formation began, were shown to be strongly autoxidizable at pH values of 6 or above. The results obtained suggest a mechanism of synthesis of humic acid-like substances in two steps, viz. (a) alteration by soil fungi of phenols synthesized by these fungi or derived from other sources (e.g. lignin decomposition) to autoxidizable phenolic compounds and (b) autoxidation of these compounds. During the latter process the phenols will react with other phenols and with amino acids or peptides to form polymers.

Martin & Haider (1969) reported that up to 33 % or more of the substances synthesized by *Stachybotrys* cultures consisted of humic acid-like compounds. The polymer yields were even greater when the fungi were cultivated on plant residues. During the growth of *Strachybotrys atra* and *Epicoccum nigrum* in bean straw or corn stalks media, most of the phenols detected were similar to those produced in synthetic glucose media; in some instances even higher concentrations were found in the straw and in the corn stalks media. In additions to the phenols of fungal origin, other phenolic compounds were found in the latter material which had probably been derived from plant residues. The results of this investigation suggested a partial degradation of the lignin and the incorporation of some of the degradation products in the 'humic acid'.

The investigations with pure cultures suggest that a large number of phenolic radicals take an active part in the formation of soil humic acids. These radicals may react with other phenols and amino compounds. The latter substances are released in soil during the breakdown of plant and microbial cells and due to excretion by living roots. The possibilities for reactions or combinations are unlimited. Dubach & Metha (1963) remarked that probably no two molecules of humic acid are exactly the same. Swaby & Ladd (1962) suggested that humic acids are three-dimensional polymers consisting of many different amino acid and phenol units without ordered sequence. According to these authors the resistance of humic acid to microbial decomposition could be explained by its occurrence as a large spherical molecule, consisting of many heterogeneous units, so that many extra-cellular enzymes from many different microorganisms would be needed to decompose it piece by piece from the outer surface. The concept of this theory is still important, although the occurrence of peptide bonds in humic acids has been proved.

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3 Influence of living plants on immobilization of nitrogen in permanent pastures

Summary*

The presence of plants has a negative effect on the net nitrogen mineralization rate in samples of turf from permanent grassland. This effect is probably caused by root excretions and dead roots which lead to the immobilization of mineralized nitrogen. When the plants of a turf sample have been killed by repeated cutting and removal of the shoots the mineral nitrogen immobilized due to the presence of plants is subsequently more readily mobilized than that immobilized in the greater part of the soil organic matter. The constant presence of plants is responsible for the accumulation of nitrogen in the organic matter of the soil of permanent grassland.

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4 The influence of living plants on mineralization and immobilization of nitrogen

Summary*

Changes in the pattern of distribution of the nitrogen of the soil and seedling grass plants have been investigated when the grass plants were grown in pots of sandy soil from a pasture, at pH 5.7. Net mineralization of soil nitrogen was not observed during an experimental period of one month in the absence of added nitrogenous fertilizer (Tabel 4.2).

Addition of labeled nitrogen (as ammonium sulphate) to the soil at the beginning of the experimental period resulted in a negative net mineralization during this period (-6 to -16 mg N per pot containing 532 g of dry soil, Table 4.4b). When none of the fertilizer nitrogen remained in its original form in the soil it was found that approximately 12 % of the labeled nitrogen had been immobilized in soil organic compounds.

Clipping of the grass at this date was followed by a decrease in the amount of labeled soil organic nitrogen, indicating that mineralization was not depressed by living plants.

The application of unlabeled ammonium sulphate subsequent to the utilization of the labeled nitrogen did not decrease the amount of immobilized labeled nitrogen in the soil organic matter, as would be expected if the organic nitrogen compounds of the soil had been decomposed to ammonia. This was thought to be due to the fact that decomposition of organic nitrogen compounds in permanent grassland results in the production of peptides, amino acids etc. which are utilized by microorganisms without deamination taking place.

In pots with ageing grass plants, labeled organic nitrogen compounds were found to be translocated from the grass shoots to the soil (Table 4.7).

Net mineralization of soil organic nitrogen was positive in the contents of pots containing killed root systems (approx. 10 mg N per pot during 1 month, Table 4.3b). About 8 % of the labeled nitrogen added to the contents of such pots, in the form of ammonium sulphate, was found to be present in soil organic nitrogen compounds approximately 4 weeks after application, while a total of about twice this amount of soil organic nitrogen was mineralized during that period.

From the results obtained in this investigation, it is concluded that the constant presence of living plants is responsible for the accumulation of nitrogen in organic compounds in permanent grassland. No evidence was obtained that the decomposition of such compounds in the soil is inbibited by living plants.

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5 Amino acid composition of humic acid-like polymers produced by streptomycetes and of humic acids from pasture and arable land

Summary*

Strains of streptomycetes, cultivated in a glycerol-nitrate medium, synthesized humic acid-like substances. With one strain 13.7 % of the NO_3-N in the medium was converted into humic acid-like substances after 59 days. Hydrolysis with 6 N HCl released 40 % of the 'humic acid' N as NH_4- and amino acid-N.

With humic acids isolated from soils of arable land and 10-year-old pasture about 60 % of the humic acid N was found to be present as NH_4- and amino acid-N in the hydrolysate.

The amino acid patterns of the hydrolysates of pasture soil, arable soil, and the humic acids of these soils were rather similar, resembling the amino acid composition of the hydrolysates of the 'humic acids' of two *Streptomyces* strains.

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*Soil Biology and Biochemistry 4(1972):339-345

6 Availability of microbial and soil organic nitrogen to a Pseudomonas strain and the effect of soil organic matter on the availability of casein nitrogen

Summary*

A microbiological method has been devised to determine the availability of microbial and soil organic N to a proteolytic *Pseudomonas* strain. The growth of the *Pseudomonas* sp. on soil fractions or on microbial material as the sole N source was compared with the growth on casein-N.

The nitrogenous constituents of 3-day-old *Arthrobacter* cells were found to be more resistent to degradation than those of 6-day-old cells during an incubation period of 4 days.

The percentage of available soil organic N and available humic acid N increased with the pH of the medium in which the availability was determinded. The percentage of available organic N in grassland soil was twice that in arable soil.

The proportion of N utilized by the proteolytic *Pseudomonas* sp. from humic acids extracted from the soil with sodium pyrophosphate (pH 7) was four times higher than that utilized from the total soil organic matter. When NaOH had been the extractant of the humic acid fraction, the relative availability of the N of the humic acid fraction was twice as high as that of the entire soil organic matter.

The availability of N of humic acid-like compounds, synthesized by a *Streptomyces* strain, to the *Pseudomonas* sp. was similar to that of humic acids extracted from grassland soil by NaOH, viz. 11 to 25 % (depending upon the pH of the culture solution) of the total N contained in the humic acid fraction.

The availability of casein-N decreased in the presence of soil or soil humic acids. This reduction of casein assimilation was partly offset by the addition of $CaCO_3$ to the test medium; it appeared to be a pH effect.

The results obtained suggest that more protein-like compounds are incorporated in the soil organic matter of grassland than in that of arable land.

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*Soil Biology and Biochemistry 4(1972):347-358

7 General discussion

The purpose of the present work was to investigate the mechanism responsible for the accumulation of soil organic nitrogen in permanent grassland. The low recovery of fertilizer nitrogen in the grass shoots is probable partly associated with this accumulation, which implies the immobilization of nitrogen in microbial cells or soil organic matter.

The soil organic nitrogen of turf samples of which the grass roots had been killed was found to be mineralizable to a higher degree than that of arable land (the content of mineral nitrogen in the pots containing approximately 600 g of dry soil increased by 45 mg and 6 mg, respectively, during two months, Table 3.1). The immobilization of labeled fertilizer nitrogen in these turf samples was greater than that in soil of arable land (from 47.6 mg labeled fertilizer nitrogen which was applied as $(NH_4)_2SO_4$ 7.3 and 0.7 mg N, respectively, were immobilized, Table 3.1). This points to the presence of larger amounts of metabolizable carbonaceous material in the turf. Disturbing and mixing the soil of the turf samples with killed roots did not promote mineralization (Table 3.3).

In the case of turf samples containing living plants, the net mineralization of soil organic nitrogen was slightly negative (approximately – 10 mg N per pot, Table 3.2). Mixing of the soil of turf samples containing living plants did not stimulate the release of soil organic nitrogen (Tables 3.7 and 3.8). Comparison of these results with the positive net mineralization rates of the turf samples with killed roots (22–24 mg N during two months, Table 3.3) indicates that growing plants are mainly responsible for the accumulation of soil organic nitrogen in grassland. In spite of this, experiments with labeled fertilizer nitrogen showed that part of the nitrogen taken up by the plants (P14) had been derived from unlabeled soil organic nitrogen (Table 4.4b). The amount of immobilized (labeled) fertilizer nitrogen (I15) was greater than P14, resulting in a negative net mineralization (P14–I15) and consequently in an accumulation of soil organic nitrogen (6–16 mg N per pot containing 532 g of dry soil). When the photosynthesis of the grass plants was reduced by clipping the shoots after the fertilizer nitrogen had been taken up, the net mineralization of soil organic nitrogen increased due to a reduced immobilization (Table 4.4b) The increased mineralization was attributed to the excretion of less carbonaceous matter by the roots of the clipped grass plants, resulting in a reduced immobilization of fertilizer nitrogen. Removal of the grass shoots by clipping may cause the death of part of the root system of the turf and may enrich the soil with more decomposable lignin-containing material. Apparently this lignin did not promote the incorporation of available nitrogen in the soil organic matter as was the case with the excretion products of roots of plants which were not clipped.

Mineralization of soil organic nitrogen was expected to proceed as far as ammonia. However, addition of unlabeled ammonium sulphate did not promote the liberation of previously immobilized labeled fertilizer nitrogen (Table 4.5). These results suggest that the decomposition of organic nitrogen compounds in pasture proceeds to such compounds as peptides, amino acids etc,. which are utilized by microorganisms without being deaminated.

Nitrogen recently immobilized in turf samples with living grass plants was mineralized more readily than soil organic matter upon killing of the grass plants. This was demonstrated in a pot experiment with turf samples dressed with labeled nitrate (Table 3.6). The plants were killed when the nitrate had been used up. Two months later 2 % of the originally present soil organic nitrogen had been liberated (N^{14}) as contrasted with 24 % of the more recently immobilized nitrogen (N^{15}).

The water content of the soil in the pot experiments was kept at 60 per cent of the waterholding capacity by addition of water twice a day. This treatment is favourable for microorganisms, but, according to Harmsen (Section 2.2.2) less favourable for humus formation. Therefore, more labeled fertilizer nitrogen might have been incorporated in soil organic matter under natural conditions where the changes in soil humidity are more extreme than those of pot experiments.

High numbers of chromogenic streptomycetes occur in pasture soil. Several strains of such organisms were tested for the ability to produce humic acid-like substances in a glycerol-nitrate medium. All the strains tested were found to produce humic substances in this medium (Table 5.1). These results suggest that microorganisms may contribute to the synthesis of soil humic acids.

Hydrolysis with 6 N HCl released less nitrogen in the form of amino acids and ammonia from humic acid-like substances synthesized by *Streptomyces* strain A (40 %) than from soil of pasture (60 %) or arable land (54 %) and from humic acids obtained from these soils (60 %), see Table 5.3. From these results it might be concluded that less protein is incorporated in the humic acid-like substances produced by streptomycetes than in the soil organic matter. Due to the fact that streptomycetes are proteolytic organisms, the concentration of protein may have been lower in the *Streptomyces* culture than in the soil during the spontaneous condensation of the building units leading to the synthesis of humic substances. No evidence was obtained that the chromogenic streptomycetes contribute more extensively to the humus formation in pasture soil than in arable soil, because the amino acid composition of the hydrolysates of both soils were rather similar (Table 5.4).

A more marked difference between the soil organic matter of pasture and that of arable land was when soil or fractions of soil organic matter were used as the only nitrogen source for the growth of a proteolytic *Pseudomonas* strain. It appeared that a larger percentage of the organic nitrogen in grassland soil was available to the *Pseudomonas* than it was the case with arable soil (Tables 6.3, 6.4, 6.5 and 6.6).

The availability to the proteolytic *Pseudomonas* sp. of nitrogen of humic acids extracted from the soil with sodium pyrophosphate (pH7) was twice as high as that of humic acids extracted with NaOH (Table 6.6). The availability of the nitrogen of the latter humic acid fraction was generally higher than that of the entire soil nitrogen (Tables 6.3 and 6.5). These results show that humic acids are not representative for soil organic matter.

The addition of $CaCO_3$ to test medium promoted the availability of soil nitrogen, apparently as a result of the higher pH value of the medium (Tables 6.3 and 6.5).

The availability to the *Pseudomonas* sp. of nitrogen of humic acid-like compounds, synthesized by a *Streptomyces* strain, varied between 11 and 25 % (depending upon the pH of the culture solution) of the total nitrogen contained in this fraction. It was similar to that of humic acids extracted from grassland soil by NaOH.

The availability of casein nitrogen was independent of the pH of the culture solution (Fig. 6.1) as contrasted with that of soil organic nitrogen which was dependent on the pH (Table 6.3). The availability of casein nitrogen was reduced in the presence of soil organic matter (Tables 6.8, 6.9 and Fig. 6.3), probably owing to the presumed inhibition of the proteolytic activity of the *Pseudomonas* strain. The reduction of the availability of casein due to the presence of soil organic matter was partly eliminated by the addition of $CaCO_3$ to the test medium, demonstrating that it was a pH effect (Fig. 6.3).

This response to low pH of the microbial decomposition of soil organic nitrogenous compounds and that of casein in the presence of soil organic matter, is a further indication that the latter contains protein-like compounds. The higher availability to the *Pseudomonas* strain of soil organic nitrogen from pasture as compared with that from arable land indicates that more protein-like material is incorporated in the soil organic matter of pasture. It is likely that these protein-like residues are decomposed after ploughing up of grassland resulting in a release of available nitrogen.

8 Samenvatting

Immobilisatie en mineralisatie van stikstof in grasland

Een onderzoek werd ingesteld naar de ophoping van stikstof in organische verbindingen in de zode van grasland. Deze ophoping is een gevolg van het feit dat de vastlegging (immobilisatie) van stikstof in organische stof de afbraak (mineralisatie) overtreft.

Voor dit onderzoek werden potproeven met zoden van grasland uitgevoerd. De zoden werden bemest met gemerkte ammoniak- of nitraatstikstof. Op verschillende tijdstippen na de stikstofgift werden stikstofbalansen opgesteld door de bovengrondse delen, wortels en grond te analyseren.

De netto-mineralisatie van de bodemstikstof in organische vorm in graszoden waarin de plantenwortels waren gedood was positief en werd niet bevorderd door het homogeniseren van de grond (tabel 3.3). Bij een poging de opbouw van de grond van graszoden te verstoren doch de planten intakt te laten, bleek dat dit geen invloed had op de opneming van stikstof door de grasplanten (tabellen 3.7 en 3.8).

De aanwezigheid van groeiende planten verlaagde de netto-mineralisatie van de organische stof van de grond tot nul (tabel 3.7); soms werden zelfs negatieve waarden verkregen (tabel 3.2). Onder deze omstandigheden bleef het gedeelte van de gemerkte kunstmeststikstof dat tijdens de groei van planten in de organische stof van de grond werd vastgelegd daarin aanwezig zolang de planten intakt werden gelaten (tabellen 3.4 en 3.8). Dit geldt uiteraard alleen voor de daar vermelde proefomstandigheden. Deze waarnemingen wijzen erop dat het vrijkomen van stikstof uit organische bodembestanddelen wordt tegengegaan door de aanwezigheid van groeiende planten.

Uit proeven met graszoden die bemest waren met gemerkte stikstof in de vorm van KNO_3 en waarvan de planten gedood waren op het moment dat geen anorganische stikstofverbindingen in de grond meer waren aan te tonen, bleek gedurende de hierna volgende periode van twee maanden de ongemerkte bodemstikstof in organische vorm minder gemakkelijk te mineraliseren dan de kort van tevoren in de organische stof vastgelegde (gemerkte) stikstof; de afneming van stikstof in organische vorm voor beide fracties bedroeg respektievelijk 2 en 24 % (tabel 3.6).

Om de spreiding tussen de resultaten van verschillende potten met dezelfde behandelingen te reduceren, werden potproeven met ingezaaid gras uitgevoerd. De grond die hiervoor werd gebruikt was afkomstig van grasland. Nadat de grond in de potten goed doorworteld was, werd bemest met gemerkte stikstof in de vorm van ammoniumsulfaat. Na ongeveer twee weken werd geen stikstof in deze vorm teruggevonden. Op dit tijdstip bleek dat minder stikstof uit de organische stof van de grond ter beschikking van de plant was gekomen (P14) dan ammoniumstikstof in de organische fractie van de grond was vastgelegd (I 15), zie tabel 4.4b. Dit betekent dat een toename van de hoeveelheid organische stikstofverbindingen in de grond had plaats gevonden. Gemiddeld werd 12 % van een stikstofgift in de organische fractie van een grond vastgelegd. Dezelfde resultaten werden een maand na toevoeging van de kunstmeststikstof verkregen. Indien twee weken na de toediening van de anorganische stikstofverbinding de bovengrondse delen van de grasplanten werden geknipt en verwijderd, bleek na nogmaals twee weken geen toename van de bodemstikstof in organische verbindingen te hebben plaats gevonden.

Toediening van ongemerkte ammoniumsultaat, nadat de gemerkte stikstof door de planten was opgenomen en in de organische stof van de grond was vastgelegd, had geen afname van de hoeveelheid gemerkte organische bodemstikstof (I15) tot gevolg (tabel 4.5). Een dergelijke afname had kunnen worden verwacht indien de afbraak van de organische stikstofverbindingen tot ammoniak zou verlopen. De verkregen resultaten doen vermoeden dat de afbraak van organische stikstofverbindingen in grasland verloopt tot produkten zoals aminozuren en peptiden die door micro-organismen weer worden geassimileerd en in celbestanddelen worden vastgelegd zonder dat ammonifikatie heeft plaats gevonden.

Tijdens het ouder worden van het ingezaaide gras werd gemerkte stikstof van de bovengrondse delen naar de grond getransporteerd (tabel 4.7). Deze verrijking van de grond met gemerkte stikstof had echter geen ophoping van stikstof in organische verbindingen tot gevolg, omdat evenveel ongemerkte bodemstikstof door de grasplanten werd opgenomen.

De positieve invloed van groeiende planten op de ophoping van organische stikstofverbindingen in de grond wordt waarschijnlijk ten dele veroorzaakt door de uitscheiding van produkten door wortels die kunnen fungeren als koolstof- en energiebron voor micro-organismen. Tijdens het afsterven van bepaalde micro-organismen kan een deel van hun stikstofverbindingen worden omgezet in humusachtige verbindingen. Aangezien veel *Streptomyces*-soorten in staat zijn tot de produktie van deze verbindingen en streptomyceten in grond van grasland een groter percentage van de totale microflora uitmaken dan in grond van bouwland, werden deze organismen onderzocht op het vermogen om humusachtige stoffen te produceren (zie hoofdstuk 5). Dit werd nagegaan in een glycerol-nitraat medium. Een stam zette 13,7 % van de nitraatstikstof om in humuszuurachtige verbindingen (tabel 5.2). Hydrolyse met 6 N HCl maakte 40 % van de stikstof van dit 'humuszuur' vrij als ammonium- en aminozuurstikstof (tabel 5.3). Bij humuszuren geïsoleerd uit grond van bouwland en van tien jaar oud grasland kwam door een dergelijke hydrolyse in beide gevallen 60 % van de humuszuurstikstof vrij als ammonium- en aminozuurstikstof.

De aminozuursamenstelling van de hydrolysaten van grond van grasland, grond van bouwland en van de humuszuren van deze gronden bleken vrijwel gelijk te zijn (tabel 5.4). Deze samenstelling toonde veel overeenkomst met die van de hydrolysaten van 'humuszuren' van twee *Streptomyces* stammen.

In hoofdstuk 6 werd een micro-biologische methode geïntroduceerd om de beschikbaarheid van stikstof in micro-organismen en die in organische bodembestanddelen te bepalen. Dit werd gedaan door de groei van een eiwitsplitsende *Pseudomonas*-soort in een vloeibaar medium met het te bestuderen materiaal als enige stikstofbron te vergelijken met de groei in een medium met caseine als stikstofbron.

De stikstofhoudende verbindingen van drie dagen oude *Arthrobacter*-cellen bleken resistenter te zijn tegen afbraak dan die van zes dagen oude cellen (fig. 6.2).

De beschikbaarheid van de in organische vorm voorkomende bodemstikstof voor de *Pseudomonas*-stam nam toe met stijgende pH van het medium waarin de beschikbaarheid werd bepaald; deze beschikbaarheid was bij grasland hoger dan bij bouwland (tabellen 6.3 en 6.5).

De eiwitsplitsende *Pseudomonas*-stam maakte procentueel tweemaal zoveel stikstof vrij uit humuszuren die geëxtraheerd waren met natriumpyrofosfaat (pH7) dan uit humuszuren die geëxtraheerd waren met natriumhydroxide (tabel 6.6). De uit de grond geëxtraheerde humuszuren waren in het algemeen beter beschikbaar dan de stikstof van de grond als geheel.

De beschikbaarheid van stikstof van humuszuurachtige verbindingen, gesynthetiseerd door een *Streptomyces*- stam, was gelijk aan die van humuszuren die met natriumhydroxide uit grond van grasland waren geëxtraheerd doch beter dan die van humuszuren uit bouwland. Afhankelijk van de pH van de voedingsoplossing werd 11 tot 25 % van de aanwezige stikstof door de *Pseudomonas* opgenomen.

De beschikbaarheid van caseinestikstof voor de *Pseudomonas*-stam was slechter zowel in aanwezigheid van grond (tabellen 6.8 en 6.9) als in aanwezigheid van humuszuren. Deze verminderde caseine-assimilatie werd gedeeltelijk opgeheven door de toedinening van $CaCO_3$ aan het testmedium; het bleek een pH-effect te zijn (fig. 6.3).

De verkregen resultaten doen vermoeden dat de organische stof van de grond eiwitachtige verbindingen bevat en dat deze verbindingen in de organische stof van grasland in grotere hoeveelheden voorkomen dan in die van bouwland.