THE INFLUENCE OF LIVING PLANTS ON DENITRIFICATION

J. W. WOLDENDORP

THE INFLUENCE OF LIVING PLANTS ON DENITRIFICATION

(MET EEN SAMENVATTING IN HET NEDERLANDS)

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWKUNDE
OP GEZAG VAN DE RECTOR MAGNIFICUS IR. W. F. EIJSVOOGEL,
HOOGLERAAR IN DE HYDRAULICA, DE BEVLOEIING,
DE WEG- EN WATERBOUWKUNDE EN DE BOSBOUWARCHITECTUUR,
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VAN EEN COMMISSIE UIT DE SENAAT
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OP WOENSDAG 11 DECEMBER 1963 TE 16 UUR

DOOR

J. W. WOLDENDORP

H. VEENMAN EN ZONEN N.V. – WAGENINGEN – 1963

STELLINGEN

I

Onder de in Nederland heersende klimatologische omstandigheden gaat minstens 10 procent van een stikstofbemesting door vervluchting verloren.

Dit proefschrift.

II

De efficiëntie, waarmee wortelexcreties door micro-organismen in celmateriaal worden omgezet, bepaalt in hoge mate het concurrerend vermogen van deze organismen in de rhizosfeer.

Dit proefschrift.

III

De dikwijls waargenomen aanzienlijke verhoging van de korrelopbrengst van winterrogge als gevolg van een late, aanvullende stikstofbemesting, is mede toe te schrijven aan de droogteresistentie van dit gewas.

W. H. VAN DOBBEN, IBS Jaarboek 1962, 77–89.

IV

Het vervangen van algemeen gebruikte „niet Nederlandse" woorden door „Nederlandse" woorden dient met voorzichtigheid te geschieden.

V

Het opstellen van een mathematisch model voor het proces van de natuurlijke regulatie van het aantal individuen in een dierpopulatie, is principieel onmogelijk.

VI

De achteruitgang van de oeverzwaluw als nederlandse broedvogel kan slechts met zekerheid worden vastgesteld onder eliminatie van het landschapselement als factor. Vooralsnog behoeven geen maatregelen getroffen te worden om het voortbestaan van de soort als broedvogel te verzekeren.

J. J. QUARLES VAN UFFORD, Proefschrift, Delft, 1963.
Ardea, 50, 71, 1962.

VII

De bestudering van de opname en verwerking van stifstofverbindingen door hogere planten dient onder steriele (axene) omstandigheden te geschieden.

VIII

Op het moment, dat nitraatvorming in de grond kan worden aangetoond, is stikstof geen beperkende factor voor de groei van planten en micro-organismen.

Proefschrift van J. W. WOLDENDORP
Wageningen, 11 december 1963.

IX

De verklaring, die ILLUMINATI en MARINO geven van het effect van het oplosmiddel op het verloop van de reakties van 2- en 4-chloorchinoline met piperidine, is onjuist.

G. ILLUMINATI and G. MARINO, Chemistry and Industry, 3, 1287, 1963.

X

Er zijn aanwijzingen, dat vitamine K bij de nitraatreduktie in bepaalde microorganismen (o.a. *Bacillus*-soorten) een functie als electronen-acceptor heeft.

Zie o.a. C. MARTIUS, The Enzymes deel 7, pag. 531.
A. MEDINA and C. F. HEREDIA, Biochim. et Biophys. Acta 28, 452–453, 1958.

XI

Het hoge nitraatgehalte van gras na een stikstofbemesting in de vorm van kalkammonsalpeter wordt veroorzaakt doordat nitraat bij aanwezigheid van ammoniak niet of slechts gedeeltelijk verwerkt wordt.

P. F. J. VAN BURG, Proefschrift, Wageningen, 1962.

XII

De conclusie van WIESEMÜLLER, dat het ruwvezelgehalte van gras verhoogd wordt als gevolg van een stikstofbemesting, is slechts in bepaalde gevallen juist.

W. WIESEMÜLLER, Z. Landw. Versuchs und Untersuchungswesen, 9, 199, 1963.

XIII

De opvatting van HUIZINGA over bord- en zetspelen, „Een volstrekt steriel kunnen, dat de geestesvermogens slechts eenzijdig scherpt en de ziel niet verrijkt, ...", is niet van toepassing op het schaakspel.

J. HUIZINGA, Homo Ludens, 4e druk, pag. 203.

VOORWOORD

Het bewerken van een proefschrift is in opzet een individuele bezigheid; het resultaat is echter de vrucht van vele vormen van samenwerking. Dit voorwoord geeft mij de gelegenheid, hen, die bij het tot stand komen van dit proefschrift betrokken zijn geweest, te bedanken.

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THE INFLUENCE OF LIVING PLANTS ON DENITRIFICATION

(met een samenvatting in het Nederlands)

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CONTENTS

CHAPTER 1

A SURVEY OF EARLIER INVESTIGATIONS ON THE NITROGEN CYCLE IN GRASSLAND SOILS, WITH SPECIAL REFERENCE TO DENITRIFICATION

A. INTRODUCTION

It is a well-known fact that microorganisms are involved in the different parts of the nitrogen cycle in the soil. The investigations performed with pure cultures of the microorganisms responsible for fixation of elementary nitrogen, immobilization and mineralization of nitrogenous compounds, nitrification, and denitrification, enabled the explanation of many phenomena observed in soils. Under field conditions most quantitative investigations of nitrogen transformations have been carried out with arable soils.

Relatively little is known about the importance of the above-mentioned processes in permanent-grassland soils. In a great number of field experiments, MULDER (74) and VAN BURG (22) have shown that on permanent pastures in the Netherlands, the recovery of fertilizer nitrogen in the grass tops seldom exceeds 60 per cent and is usually less than 50 per cent. Similar results were obtained in other countries under different climatological conditions.

The low recovery indicates that, unless there is a considerable storage in the roots, about half of the added fertilizer nitrogen disappears because of processes, not involving the plants.

This disappointing behaviour of permanent grasslands provided the motivation for the present investigation which endeavours to trace the part of the nitrogen unaccounted for in the tops. In general terms, the nitrogen may escape from the plant in different ways. Apart from being stored in the roots, it may be immobilized in microorganisms and soil-organic matter. In the case of a nitrate dressing, nitrogen may be leached or volatilized because of denitrification. When supplied in the ammonia form, chemical fixation by soil-organic matter and clay minerals may occur. Nitrification and subsequent denitrification have also to be taken into account. From alkaline soils ammonia may volatilize directly.

In the present investigation an attempt has been made to clarify the ways by which some of the above-mentioned processes succeed in eliminating such a large proportion of a nitrogen dressing. Since losses by volatilization were found to be important, much attention has been payed to the role of denitrification in permanent grassland.

B. LITERATURE ON NITROGEN TRANSFORMATIONS IN GRASSLAND SOILS

1. *General features of the nitrogen cycle in permanent-grassland soils*

The main difference between soils under permanent grassland and arable soils is that the former is always occupied by the crop, while the latter is periodically disturbed by cultivation alternating with periods of cropping. This has some very important consequences as far as the nitrogen cycle is con-

cerned. Earlier literature on the subject has been discussed thoroughly by HARMSEN and VAN SCHREVEN (47) in an excellent review on the mineralization of organic nitrogen in soil.

As early as 1885, LAWES and GILBERT (67) showed that a large accumulation of organic residues, and consequently of nitrogen, may occur in pastures. This observation has been verified by a large number of other investigators. Figures on the organic-matter content of soils covered by permanent grassland, under conditions occurring in the Netherlands, have been presented by 'T HART (49). Climatological conditions, ground-water level and soil type greatly influence the quantity of organic matter finally accumulated; a large proportion of clay particles, for instance, causes high organic-matter contents (49, 91). During ageing of the grassland, the organic-matter content gradually increases, until finally an equilibrium is reached between mineralization and supply of fresh organic matter. Few exact data are available in the literature concerning the time required to reach this stage. RICHARDSON (91) found equilibrium in grasslands occurring in the vicinity of Rothamsted, only after more than 150 years of use as such. Fertilizer nitrogen and grassland management seem to have a marked influence on the process. With heavy nitrogen dressings on orchard grassland under a mulching system, equilibrium was found to be reached after 25 years (87). Nevertheless, it may take many decades before equilibrium between mineralization and immobilization of nitrogen is obtained, involving accumulations of organic matter of more than 10–15 per cent in the surface layers. Although the supply of plant residues in grassland soils may exceed that in arable soils (65), the accumulation is mainly the consequence of a delay in the breakdown of organic matter.

The slow decomposition of plant residues in permanent grassland and in other soils under permanent vegetation is not completely understood. HARMSEN (46) applies the theory of ENDERS (28) on humus formation to the situation occurring in grassland soils. This theory is based on an accelerated synthesis of humus under unfavourable soil conditions. According to HARMSEN, conditions in the surface layer of permanent grassland are promoting the synthesis of humus, i.e. large fluctuations in temperature, moisture and irridation. Recent investigations of BIRCH (10), however, show that the drying and remoistening of a soil stimulates the breakdown of organic matter. Generally, an alternation of the soil conditions increases mineralization. Therefore, it is likely that the continuous mixing of arable soils brings about a rapid breakdown of organic substances, whereas in all undisturbed soils, like permanent pastures, breakdown is retarded. The fact that ploughing up of grassland is followed by a rapid decline of the organic matter content (49), supports this hypothesis.

A second important difference between permanent pastures and arable soils, observed by several investigators, is that, broadly, the mineral-nitrogen content of grassland soils is considerably lower than that of comparable arable soils. In the latter, mineral-nitrogen content shows a rise during spring and autumn, while that of grassland is invariably low during the whole year. RICHARDSON (91) found values for ammonium nitrogen between 2 and 8 p.p.m., and for nitrate nitrogen between zero and 2 p.p.m. in three subsequent years. Such values are common for permanent pastures.

After an addition of fertilizer nitrogen, the mineral-nitrogen content declines very rapidly. RICHARDSON (91) observed that in early spring half of the

nitrogen disappeared within a week, while in late spring only a few days were required for its disappearance.

It is not clear whether or not the low quantities of ammonium nitrogen reported may be regarded as real values. In most cases ammonia was estimated by distillation with $Mg(OH)_2$ which may have brought about some decomposition of organic nitrogenous compounds.

The low nitrate level in permanent pastures may be explained by an observation of JANSSON (55), namely that nitrification of ammonium nitrogen only occurred when its rate of production was faster than its rate of removal by other processes like immobilization and uptake by plants. This situation is not met in grassland soils. A different explanation of the occurrence of a low nitrate content in grassland was given by LYON and BIZELL (68), who suggested that plant roots may suppress nitrification, although JENSEN (57) and others have shown that nitrification is not depressed by organic substances. SOULIDES and CLARK (104) recently demonstrated that nitrification in grassland was indeed retarded. Similar observations were made by CUNNINGHAM and COOK (27). However, some of these investigators did not account for the nitrate taken up by the plant. Realizing this, VAN BURG (22) found that relatively large quantities of nitrate accumulated in the grass tops; despite this no nitrate was detected in the soil.

As stated already by RICHARDSON (91), leaching of nitrate from grassland soils during spring and summer is generally of no importance. It may occur to some extent only in light sandy soils after heavy rains. This was recently confirmed by HARMSEN and KOLENBRANDER (unpublished results). During the winter the situation is different: leaching of nitrate and even of ammonia may occur.

Recently, claims have been made of significant gains in the nitrogen content of grassland soils by processes other than symbiotic nitrogen fixation (82, 87, 107). As most of these results have been obtained in field experiments, the claims are not convincing. Moreover, nothing is known concerning the mechanism of these gains. Nitrogen fixation by azotobacters does not come into account.

In summary, it can be stated that the nitrogen cycle in grassland soils is characterized by:

1. an accumulation of large quantities of organic matter,
2. the presence of very low quantities of mineral nitrogen, especially of nitrate nitrogen, throughout the year,
3. a rapid desappearance of added fertilizer nitrogen,
4. a suppressed nitrification.

The mechanisms underlying these characteristics are imperfectly understood.

2. *Disappearance of fertilizer nitrogen in permanent pastures*

As stated above, little is kown concerning the rapid disappearance of fertilizer nitrogen in grassland soils. In view of the attention paid to this part of the nitrogen cycle in the present paper, it is worthwhile to subject to a detailed examination the different mechanisms by which nitrogen may be removed.

a. Nitrogen Turnover. Obviously a large proportion (50–60%) of the fertilizer nitrogen applied in spring or summer is taken up by the plants. As

leaching in this season may be excluded, HARMSEN and VAN SCHREVEN (47) suggest that much of the remainder is immobilized in the organic matter of the soil. Unfortunately, the quantity of nitrogen contained in the soil organic matter is usually too high to estimate, by means of conventional methods, small gains in organic nitrogen following an addition of fertilizer nitrogen. However, by applying ^{15}N-labeled nitrogen, it is possible to trace fertilizer nitrogen upon its application on grassland.

With respect to the hypothesis of HARMSEN and VAN SCHREVEN, two mechanisms may be considered. Firstly, mineral nitrogen may be incorporated directly into organic compounds by microorganisms. In most grassland soils, there is a surplus of carbogeneous material. Microorganisms may use these compounds in combination with mineral nitrogen to build up their cell material. This type of immobilization gives rise to a net increase in soil organic nitrogen. However, it is not possible to trace the quantities of nitrogen immobilized in this way simply by adding ^{15}N-labeled fertilizer to permanent grassland, and then estimating the ^{15}N-content of soil organic matter when all mineral nitrogen had disappeared. For, as was clearly shown by JANSSON (55) and other investigators, a constant mineralization of soil organic nitrogen will occur in any soil. The energy liberated during the breakdown of soil organic matter is used by the microorganisms to immobilize anew part of the mineralized nitrogen. This continuous mineralization-immobilization cycle of nitrogen has been called "Nitrogen Turnover".

When the mineralized nitrogen is diluted by adding (^{15}N-labeled) fertilizer nitrogen, microorganisms do not differentiate between these two mineral nitrogen sources in re-immobilization. Therefore, part of the fertilizer (^{15}N) is always found in the organic-nitrogen fraction of the soil. A relative shortage of carbogeneous compounds results in an increase of total mineral nitrogen (although still label is incorporated into soil organic matter), while a surplus of carbogeneous compounds results in a decrease of the total mineral-nitrogen level. To measure the surplus immobilization of mineral nitrogen, the level of the latter has to be known. When mineral nitrogen is being removed by other processes (e.g. uptake by plants), surplus immobilization can not be measured. Consequently, the quantities of labeled nitrogen in soil organic matter *per se* do not provide a decisive answer concerning a direct surplus immobilization of fertilizer nitrogen.

The second mechanism by which fertilizer nitrogen may turn into soil organic matter, is by passing the plant. After incorporation in the nitrogenous compounds of the plants, root excretions and dying roots may enrich soil organic matter with nitrogen originating from the fertilizer. Removal of grass tops by cutting and grazing may cause the death of part of the root system and thus promote the formation of soil organic matter.

Since the quantity of label in the soil in experiments with ^{15}N-labeled fertilizer may be the result of both processes, it is difficult to decide which process prevails. When no gains or losses of soil nitrogen other than by plant uptake are involved, it is possible to estimate the increase in soil nitrogen at the end of an experiment. As the immobilized nitrogen is a mixture of mineralized soil nitrogen and fertilizer nitrogen, so the nitrogen taken up by the plants is a mixture of both nitrogen sources. The amount of nitrogen in the plant not derived from the fertilizer, therefore, represents the mineralized nitrogen. When, at the end of an experiment, the amount of fertilizer nitrogen in the

soil organic matter exceeds the amount of soil nitrogen in the plant, a net increase in soil nitrogen has taken place. There is a lack of experiments on this subject performed with permanent pastures. However, it is possible to apply the above analysis to data given in a paper by WALKER (119) who describes a pot experiment with sown grass and clover, in which different quantities of nitrate and ammonium nitrogen were applied. Neglecting the small amount of nitrogen in the seed, calculations show that, with grass, the highest nitrogen applications cause an increase of soil nitrogen. The gains after a nitrate dressing exceed those after an ammonium application (Table 1). The quan-

TABLE 1. Increase in organic soil nitrogen after the addition of labeled nitrate and ammonium nitrogen to sown perennial ryegrass (data of Walker).

Mg N applied per pot	N source	Mg soil N in the grass (WALKER, Table 3)	Mg fertilizer N in the soil (WALKER, Table 4)	Difference = increase in soil N
		Total N — Fert. N = Soil N		
99.2	NH_4^+	67.9 — 51.4 = 16.5	16.0	— 0.5
91.2	NO_3^-	47.4 — 36.1 = 11.3	25.0	+ 13.7
198.4	NH_4^+	145.6 — 123.1 = 22.5	27.0	+ 4.5
182.3	NO_3^-	123.2 — 108.2 = 15.0	29.0	+ 14.0

tities of soil nitrogen in the grass also show that, after a nitrate dressing, exchange of fertilizer nitrogen with soil nitrogen is smaller than after a dressing with ammonium nitrogen. This is in accordance with the results of an investigation by JANSSON (54), who demonstrated that soil microorganisms prefer ammonia to nitrate as a nitrogen source. If both compounds are available (e.g. after a nitrate dressing), mineralized ammonia is re-immobilized by preference. Therefore, the experiments of WALKER suggest that, after a nitrate dressing, enrichment of soil organic nitrogen is mainly caused by nitrogen which has passed the plants and is excreted afterwards. In the experiments of WALKER with clover, much larger quantities of labelled nitrogen were recovered in the soil organic matter (e.g. 78 mg after an addition of 182.3 mg nitrate nitrogen). It is impossible, however, to decide whether this quantity of labeled soil nitrogen represents a gain in total soil nitrogen, because the fixation of atmospheric nitrogen interferes with the calculations.

b. Chemical fixation of ammonium nitrogen. In the case of the chemical fixation of ammonium nitrogen by clay minerals, no fundamental difference exists between grassland and arable soils. Soil ammonium that is not removed by extraction with 1 N KCl or 1 N K_2SO_4 is generally considered to be fixed chemically. It has been pointed out by ALLISON (4) and JANSSON (55) that large differences exist between different soils, high amounts of montmorillonite and illite giving rise to a high fixation. Drying of the soil after the addition of ammonium enhances the fixation. In certain soils, especially subsoils, a large proportion of an ammonium dressing may be fixed. In surface soils according to BREMNER (16) 3–8 per cent of the ammonium content is in the fixed form. Therefore, ammonium fixation theoretically may play a significant role after an addition of fertilizer ammonia. It is likely, however, that the ammonium-fixing capacity of the soil becomes soon saturated, and after a few years plays no further role in removing fertilizer ammonium.

c. Volatilization of fertilizer nitrogen. Large quantities of fertilizer nitrogen may be lost by volatilization. The evaporation of ammonia from alkaline soils is a well-known phenomenon and will certainly occur in the appropriate grassland soils. Concerning the losses caused by denitrification, comparatively few data are available from experiments under natural conditions. In his review on soil-nitrogen balances, ALLISON (6) stated that few losses usually occur in soils with sod crops. This conclusion was based on the analysis of a number of lysimeter experiments. According to ALLISON, uptake by plants will prevent denitrification. WALKER (118), in his experiments with permanent grassland, comes to the same conclusion. The results derived from lysimeter experiments, however, are open to serious criticism. The nitrogen balances obtained with lysimeters are for the most part the results of experiments continuing for more than five years. As there have been many claims made of nitrogen gains in permanent grassland, (also summarized by ALLISON), the observed closed nitrogen balances may be the result of an equilibrium between denitrification losses and gains caused by fixation of atmospheric nitrogen. With high fertilizer dressings, nitrogen fixation may be depressed. Denitrification losses, however, may be expected to be higher (it takes more time before all nitrogen will be removed by the plants). Indeed, most of the lysimeter experiments performed with high nitrogen dressings show deficits, while after the application of small doses of fertilizer, gains are observed. Recently, similar results were obtained by JANSSON (56), who found losses at high nitrogen applications in pot experiments continued for several years, whereas small gains occurred upon the addition of small doses of fertilizer nitrogen. This clearly demonstrates that lysimeter experiments can give no impression of the magnitude of denitrification losses before more is known about the occurrence of nitrogen gains.

In his experiments on the influence of the ground-water level on nitrogen yields in the grass tops of permanent pastures, MINDERHOUD (73) obtained, in one of three subsequent years, a correlation between ground-water level and nitrogen yields. Although no nitrogen balances are presented, the low recoveries at a high water level suggest that denitrification may have been involved. Unfortunately no impression of the magnitude of the denitrification can be obtained from these experiments.

The most accurate method of acquiring information concerning denitrification, is by means of short term experiments with labeled nitrogen. In the previously noted experiments of WALKER (119) with sown grass and peas, losses of about 25–35 per cent were observed, with both ammonium and nitrate nitrogen. In the case of nitrate, volatilization as a result of denitrification is the most likely explanation. At the beginning of the experiment, the ammonium dressing was mixed with $CaCO_3$, which may have resulted in direct volatilization of ammonia. Nitrification followed by denitrification also may have been the cause of the observed losses. WHEELER (120) has shown that nitrification and denitrification may occur simultaneously under apparently aerobic conditions.

The few data available on this subject clearly demonstrate that volatilization may be one of the most important causes of the low nitrogen recovery in the grass tops.

C. Literature on denitrification in soils

1. *Introduction*

Most investigators dealing with denitrification in soil concentrate on the microbial reduction of nitrate to gaseous products, which gives rise to losses in soil nitrogen. Although the process is an indispensable chain in the nitrogen cycle, it is also an alarming one, for it provides a pathway for fertilizer and soil nitrogen losses. In 1886 the occurrence of these gaseous reduction products of nitrate was first studied by Gayon and Dupetit (38), and since that time a large number of investigations has dealt with denitrification in order to elucidate the conditions under which it occurs, and to establish its importance in soils. As to the latter, different opinions were held during the first decades of this century. Nowadays it is generally believed that, under certain conditions, denitrification may bring about serious losses of nitrogen.

Literature on denitrification has been reviewed by Broadbent (18), Verhoeven (115), Nommik (79), Wyler and Delwiche (126), and Charter and Allison (24), while the biochemical aspects have been discussed by Fewson and Nicholas (34), and Nason (78).

2. *Influence of oxygen*

It is a well-known fact that during denitrification, nitrate is used by certain microorganisms as a terminal electron acceptor, resulting in a dissimilatory reduction which gives rise, along with some other products, to N_2 and N_2O. In this respect, nitrate is able to replace oxygen. Therefore, certain unsuspectedly aerobic bacteria, like *Ps. aeruginosa* and *M. denitrificans,* are able to grow under anaerobic conditions in the presence of nitrate. With *Vibrio succinogenes,* however, nitrate may replace fumarate and malate as a hydrogen acceptor, while oxygen is used as electron acceptor only when its concentration is below 2 per cent (123). Some *Clostridium*-species also possess a dissimilatory nitrate-reducing capacity (125). It seems feasible to regard nitrate as one of various electron acceptors which may be used by microorganisms, not merely as a substitute for oxygen.

When both oxygen and nitrate are present in the medium, the former is preferred. In experiments with *Ps. denitrificans,* Skerman and Mac Rae (99, 100) observed that concentrations of dissolved oxygen above 0.2 p.p.m. suppressed dissimilatory reduction of nitrate completely. With *Achromobacter liquefaciens,* however, the same investigators showed that nitrite was used as an electron acceptor in the presence of higher levels of dissolved oxygen (101). The same seems to hold for *Bacterium denitrificans* (60). In pure-culture studies, aerobic denitrification was also claimed by Meiklejohn (71), Korsakova (66) and Verhoeven (115), but since no estimations of dissolved oxygen were recorded in these investigations, the results are open to criticism.

From the above-mentioned pure-culture studies it is not possible to decide, whether or not denitrification in soil will occur in the presence of oxygen, dissolved in the soil solution. Therefore, many investigations have been carried out in order to study denitrification in soils considered as "well aerated". The results were conflicting. Broadbent (18) observed denitrification at all oxygen levels in the gas phase above the soil, while Broadbent and Stojanovic (19) reported that denitrification was inversely related to partial pressure of oxygen but still occurred at the highest oxygen levels. Bremner and Shaw

(15), NOMMIK (79), and CHARTER and ALLISON (24), however, showed that under their experimental conditions, denitrification in well-aerated soils was of minor importance. Recently, GREENWOOD (41) showed that denitrification in soil will occur only at levels of dissolved oxygen, similar to those estimated by SKERMAN and MAC RAE (99) for *Ps. denitrificans*.

The experiments concerning the influence of oxygen on denitrification in soil, have all been carried out by incubating the soils in the laboratory at different partial pressures of oxygen in either the gas mixture above the soil surface or the gas phase of the soil. An exception to this procedure was made in the experiments of GREENWOOD (41), who estimated the quantity of oxygen dissolved in the soil solution. With the former procedure no equilibrium between oxygen in the gas phase and oxygen dissolved in the soil solution may be expected. The quantity of dissolved oxygen is controlled by oxygen consumption (plants, microflora, microfauna), diffusion rate, partial pressure of oxygen in the soil atmosphere, and temperature. The partial pressure of oxygen in the soil atmosphere in turn is decreed by the partial pressure in the free atmosphere, the rate of diffusion and the consumption (referred to as the "activity" of the soil) (8). The soil conditions (moisture level, package of soil particles, occurrence of aggregates), and depth of the active layer also influence partial oxygen pressure of the gas phase. The latter is therefore one of several factors, each influencing the oxygen availability in the soil solution, and thus its effect may be completely overshadowed by other components. This explains the different results obtained by various investigators, who studied the influence of oxygen on soil denitrification. It is also apparent that conditions may vary at different points in the soil.

In addition, it may be mentioned that most investigators have worked with soils, which were previously air-dried. The experiments of BIRCH (10), however, demonstrate that re-moistening of a dried soil results in an increased oxygen uptake, thus favouring the occurrence of anaerobic conditions.

High moisture levels, additions of fresh organic materials, and high temperatures all have a diminishing influence on dissolved oxygen; therefore denitrification is often observed when one of these conditions is met. To sum up, it can be stated that under soil conditions prevailing in normal agricultural practice, the required levels of oxygen deficiency will often occur.

3. *Influence of hydrogen donors*

Although oxygen deficiency is required, no denitrification will occur when suitable hydrogen donors are lacking. While much attention has been paid to the former point, relatively few investigations have been carried out on the influence of hydrogen donors on denitrification. JANSSON and CLARK (53), incubating different substrates with nitrate under aerobic conditions, found a promoting effect of lucerne and peptone as compared with straw and glucose. This stimulatory effect, however, may be attributed to a rapid oxygen consumption as well as to the influence of different hydrogen donors. BREMNER and SHAW (15) observed that readily decomposable materials strongly stimulated denitrification (e.g. cellulose rather than lignin, wheat straw rather than water-extracted wheat straw). The same investigators did not detect denitrification when levels of native soil carbon were less than 1 per cent (15). MC GARITY (37) also found a close relationship between denitrification and quantities of native organic soil carbon. However, even at low levels, denitri-

fication was still significant. Additions of readily decomposable materials markedly stimulated denitrification; only at high contents of native soil organic matter was the influence small. It is not clear which fraction of soil organic matter may serve as hydrogen donor nor what the influence is of the C/N ratio and other factors.

In a recently published survey on the nutritional requirements of pure cultures of denitrifying bacteria, VALERA and ALEXANDER (113) showed that some species, like *Ps. aeruginosa* and *Achromobacter* spp., were able to denitrify in a glucose-nitrate medium, while others, like *Ps. stutzeri* and *B. licheniformis,* required ammonium nitrogen or amino acids in addition. Since it is not known which species are chiefly involved in soil denitrification, these data do not provide information concerning the hydrogen-donor requirements in the soil.

4. *Influence of other factors*

The influence of environmental factors on denitrification in soil has been studied by BREMNER and SHAW (15), WYLER and DELWICHE (126), and NOMMIK (79). These investigators showed that pH influenced the denitrification rate and the relative proportions of N_2 and N_2O formed. Below pH 6 denitrification was markedly retarded, N_2O being the main product.

The optimum temperature for denitrification in soil appeared to be about 60°C (see references 15, 79). Since the experiments of NOMMIK (79) were performed under aerobic conditions, temperature had influenced oxygen solubility as well. Diffusion rate of gaseous denitrification products is also stimulated by increasing temperatures. The reported high optimum temperature, therefore, may have been caused indirectly by secondary conditions. Moreover, a similar high temperature optimum has never been observed with pure cultures of the common denitrifying organisms.

It is not known if differences in environmental conditions bring about changes in the denitrifying population or merely result in an alteration of the relative proportions of N_2 and N_2O produced by the same species.

5. *Losses other than by denitrification*

The large losses of nitrogen, often observed in lysimeter, field, and pot experiments, do not always seem to have been caused by denitrification. Investigations of SOULIDES and CLARK (104), SMITH and CLARK (102), CLARK *et al.* (25), TYLER and BROADBENT (112), and GERRETSEN and DE HOOP (40) have shown that nitrite is easily decomposed chemically. This decomposition seems to be important especially during nitrification of ammonium in alkaline soils subsequently becoming acid. The mechanism of decomposition is still obscure. According to ALLISON and DOETSCH (5), the VAN SLYKE reaction is not involved. GERRETSEN and DE HOOP (40) came to the opposite conclusion. TYLER and BROADBENT (112) observed N_2 as being the main product formed between pH 5.5 and 6.5, while NO occurred only in traces.

In summing up, it may be stated that losses by volatilization may occur in soil under the influence of the following processes: 1. volatilization of ammonia in alkaline soils; 2. denitrification of nitrate or nitrite; 3. chemical decomposition of nitrite.

CHAPTER II

ANALYTICAL METHODS

A. INTRODUCTION

The investigations described in this thesis deal with soils, plants and pure cultures of micro-organisms. Although considerable variation in experimental procedures occurred, in most experiments the distribution of nitrogen had to be estimated quantitatively. The analytical methods used are described in this chapter.

B. DETERMINATION OF NITROGENOUS COMPOUNDS

1. *Ammonia in soils*

The general procedure in the estimation of ammonium nitrogen in soils consists of shaking the soil with a certain amount of an extracting agent, distillating the extract under alkaline conditions and then estimating the ammonia in the distillate by titration or Nesslerization. There are some difficulties in using this procedure. It is impossible to extract quantitatively the ammonium chemically fixed in clay minerals. It is assumed that extraction with 1 N solutions of potassium salts removes the ammonium which is not chemically fixed (55). By repeated extraction with 0.2 N H_2SO_4– 1 N NaCl-solution, part of the fixed ammonium is also removed. However, especially in grassland soils, more of the soil organic matter goes into solution by extraction with dilute acid solutions than with neutral extractants. During alkaline distillation, part of the dissolved organic nitrogen is split off and estimated as mineral ammonium. Therefore, extraction with 1 N K_2SO_4 was chosen for the determination of ammonium nitrogen.

The following procedure was adopted. 100 g soil of known moisture content were shaken mechanically with 250 ml 1 N K_2SO_4-solution for 1.5 hours. After removal of the soil by filtration, ammonium was estimated by two different methods.

In the first method two 100 ml samples of the filtrate were pipetted into 500 ml Kjeldahl flasks, (pH was fixed at 6.3 with 2 N NaOH using methyl red as an indicator), then 150 ml distilled water were added, followed by 50 ml borate buffer, (containing 8 g boric acid + 40 g Na_2BO_3 per litre), (see reference 13). Ammonia was distilled into 5 ml of a 2 per cent boric acid solution and then titrated with 0.02 N H_2SO_4 using methylred-bromocresolgreen as an indicator.

In the case of ^{15}N-samples, the ammonia was subsequently distilled into 0.1 N HCl. After conversion of the ammonium into N_2, the ^{15}N-content was estimated by means of a Metropolitan Vickers mass spectrometer.

The second method depended on the Conway-technique. Duplicate 2 ml samples of the soil extract were pipetted into Conway vessels. Upon the addition of 1 ml of a 12 percent MgO-suspension, the ammonia was distilled into 2 ml of a one per cent boric acid solution by incubation at 28 °C. Finally the ammonium was titrated with 0.005 N H_2SO_4. In general a good agreement between both methods was obtained.

2. *Ammonia in culture solutions of bacteria*

When large quantities of culture solutions were available, they were treated in the same way as the soil extracts. After removal of the cell material by centrifugating, duplicate 10 or 50 ml samples were diluted to 250 ml, followed by distillation with borate buffer.

In the experiments with resting cells, however, small quantities of ammonia had to be estimated. In this case, a somewhat modified procedure was employed. After being centrifugated, the culture solution was freed from protein with trichloroacetic acid and kaolin. Then 1 ml aliquots were pipetted into Conway vessels, followed by distillation of the ammonia into 4 ml 0.005 N HCl. Ammonium was estimated in the distillate by Nesslerization. It was possible to recover quantities of ammonium ranging from 0.5 to 20 μg accurately.

3. *Nitrate*

Since the extraction of nitrate from soils offers no difficulties, determinations were always performed in the extracts prepared for the ammonium determination. After distillation of the ammonia the samples were concentrated in the same Kjeldahl flasks to 100 ml. Then 10 ml of a 50 per cent NaOH-solution were added and the concentrating procedure was continued until a final concentration of 40 ml was reached. During the latter procedure, ammonium

formed by breakdown of soil organic matter was removed. In the concentrated solutions, nitrate and nitrite were estimated by reduction with $FeSO_4$ and Ag_2SO_4 according to the method of COTTE and KAHANE (26). When nitrite nitrogen was present, it had to be substracted from the figures obtained by the COTTE and KAHANE procedure.

Nitrate was also determined spectrophotometrically by the xylenol-sulphuric acid method of BALKS and REEKERS (7). Color development was measured with a BECKMAN DV spectrophotometer at 430 mμ. Since chloride interferes with the determination, it was necessary to use K_2SO_4 in preparing soil extracts. In the experiments with washed cells, nitrate was always determined by the xylenol method.

4. *Nitrite*

This compound was estimated according to the GRIESS-ROMEIN-VAN ECK method (72). Color development was measured with a BECKMAN DV spectrophotometer at 530 mμ. The method proved to be not entirely reliable. In the presence of amino acids, nitrite was not recovered quantitatively. Losses of about 2.5 per cent were sometimes observed. The lowering effect of amino acids on the nitrite recovery was ascribed to the VAN SLYKE reaction. No attempts were made to overcome this shortage of the GRIESS-ROMEIN method.

5. *Total nitrogen content of soils*

Estimations of the total nitrogen content of soils were performed by the KJELDAHL method. As the complete homogenization of grassland sods was not realized, five samples of 15 g each were taken for the analysis. Standard deviations were calculated. The digestion was carried out with conc. H_2SO_4 and a Se, $CuSO_4$, K_2SO_4 mixture, according to BREMNER and SHAW (14, 16). When nitrate and nitrite were present, reduction with reduced iron-powder was carried out prior to digestion (14). After digestion, ammonia was distilled with steam into 10 ml of a 4 per cent boric-acid solution. The ^{15}N-content of the samples was estimated by distillation of the ammonia from the boric-acid solution into 10 ml 0.1 N HCl, followed by conversion into N_2.

In order to examine to what extent total nitrogen of samples, without nitrate, was determined by the KJELDAHL method, as compared with the DUMAS method, samples of both soil and herbage were analysed (Table 2). Both methods gave more or less similar results except with arable sandy soil and with herbage. KJELDAHL analyses performed by the Central Laboratory of the States Mines, also where the DUMAS determinations have been carried out, corroberated with the KJELDAHL analyses reported in the present investigation. Since the KJELDAHL method proved to be not inferior to the DUMAS method with grassland soil and herbage, it was assumed to be sufficiently reliable for obtaining total-nitrogen balances.

TABLE 2. Nitrogen content (% nitrogen) of different soil and herbage samples determined with the Kjeldahl and the Dumas methods.

Sample	Kjeldahl[1] without reduction	Kjeldahl[1] with reduction	Kjeldahl States Mines	Dumas
Peat	1.16	–	–	1.28; 1.14; 1.22
Arable sandy soil	0.08	0.08	0.08	0.093; 0.089
Grassland sandy soil	0.30	0.30	–	0.291; 0.295
Herbage	1.85	1.85	1.85	1.71; 1.73

[1] Mean values of ten estimations.

6. *Total nitrogen content of herbage and roots*

Immediately after cutting, the herbage was dried under forced aeration; the roots were treated in the same way after separation of the soil. The dried material was weighed, ground and analysed by digestion of duplicate samples with conc. H_2SO_4 and a 30 per cent H_2O_2-solution. The latter was added until the solution remained clear. Ammonia was estimated by distillation with steam into 2 per cent boric-acid solution. Simultaneously with the nitrogen analysis, the moisture content in the samples was determined anew.

After the first year's experiments, this method proved to be not entirely reliable, particularly in the presence of nitrate. Thus, in subsequent experiments, reduced-iron powder was applied to reduce nitrate prior to digestion with the H_2SO_4, Se, $CuSO_4$, K_2SO_4 mixture.

The total nitrogen content of bacteria was estimated by digestion with sulphuric acid and the selenium mixture in the same way as with herbage and roots.

C. Other procedures

1. *Soil treatment in establishing nitrogen balances in grass sods*

At the end of the experiments the sods were weighed, packed in plastic bags, and stored at −18 °C until analysis. After having been thawed, the samples were rewetted to their original weight, mixed thoroughly and weighed again to determine moisture loss during the mixing procedure. Then the total nitrogen content of the sods was estimated according to the Kjeldahl method as described above.

2. *Separation of roots from soil*

The remaining part of the sods, that was not used for the Kjeldahl analysis, was washed carefully with tapwater. The water used for the washings was drained through a 56 mesh sieve in order to recover small roots. The roots were stirred mechanically in a water container to remove adhering soil particles. Then they were successively sieved, dried, ground and suspended in carbon tetrachloride to separate organic matter from heavier soil particles. Finally the roots were separated, dried, weighed and analysed for total nitrogen and ^{15}N.

The large amounts of water and carbon tetrachloride used, were concentrated. Determinations of total nitrogen in the concentrates showed that the nitrogen loss was less than 0.5 per cent of the total nitrogen content of the roots.

Although it is not possible to separate dead and living roots, this method gives a good impression of the total root mass and the nitrogen content of the roots. By subtracting the nitrogen content of the roots from that of the total sod, the nitrogen content of the soil was obtained.

3. *Determination of mineral nitrogen in the soil*

After the samples for the total nitrogen determination had been taken, three samples of 100 g each were extracted with 1 N K_2SO_4 and analysed for nitrate, nitrite and ammonium nitrogen. The moisture content was estimated by taking duplicate soil samples.

CHAPTER III

PRELIMINARY EXPERIMENTS WITH SODS FROM PERMANENT GRASSLAND

A. Introduction

The recovery in the herbage of nitrogen applied to permanent grassland usually does not exceed 50–60 per cent (Chapter I). The high quantity of nitrogen already present in the soil makes it impossible to trace the remainder of the fertilizer by conventional methods. By using labeled nitrogen, however, more information can be obtained concerning the fate of the missing part of the fertilizer.

Therefore, preliminary investigations were started in 1958 by adding ^{15}N-labeled nitrate to permanent-grassland sods placed in pots. An attempt was made to get an impression of the distribution of the added fertilizer nitrogen among herbage, roots and soil, imitating natural conditions as closely as possible. From the values obtained, a balance sheet could be drawn up. Since leaching was prevented, the quantity of nitrogen not accounted for gives an impression of the losses by volatilization. The experiments were carried out in collaboration with Ir. K. Dilz, detached as a scientific worker of the Netherlands Nitrogenous Fertilizer Industry to the Laboratory of Microbiology.

B. Materials and methods

Sods, 11 cm in diameter and 7 cm thick, were obtained from permanent grassland on peat, clay and sandy soil with a specially constructed auger. Some characteristics of these soils are summarized in Table 3. The sods were placed in pots without holes, which were dugged in to the level of the lawn in the laboratory garden. They were sheltered with plastic caps during rainy weather to prevent overflow. Temperatures were recorded both in the lawn and in the sods of each soil type.

Table 3. Some characteristics of the soils used in the preliminary investigations.

Soil type	Fresh weight g per pot	Dry weight g per pot	Waterholding capacity in % of dry matter	N-percentage in dry matter	pH
Peat	575	160	73	2.0	6.1
Clay	700	470	36	0.5	7.4
Sandy soil	700	630	18	0.4	5.2

In order to compare grass growth in the pots with that on the same soil under natural conditions, plastic cylinders of the same diameter and height as the pots were driven into the sandy soil pasture. The grass inside these rings, which was in contact with the subsoil, was treated in the same way as that in the pots.

The experiments were carried out under high-moisture conditions, as such conditions, favouring denitrification, prevail on many pastures in the Netherlands during a wet summer. Two different moisture levels were applied in the pots, corresponding with 80 and 95 per cent of the waterholding capacity (W.H.C.). The pots were weighed once or twice a day and the water content was adjusted with distilled water.

Each series consisted of 5 pots. All pots received 0.3 g of superphosphate and 0.3 g of a mixture of potassium and magnesium sulphates as a basic treatment. Nitrogen, supplied as a potassium-nitrate solution, was added as follows:

Clay and Sandy soil	*Peat soil*
2 pots: 190 mg ^{14}N + 50 mg ^{15}N	1 pot: 160 mg ^{14}N + 80 mg ^{15}N
1 pot: 240 mg ^{14}N	2 pots: 240 mg ^{14}N
2 pots: no nitrogen	2 pots: no nitrogen

Sufficient ^{15}N was added to assure a measurable ^{15}N-excess in all fractions when analysed at the end of the experiment. The rings in the field were supplied with 240 mg ^{14}N. The grass inside the rings received additional water on dry days to maintain a high moisture content. The nitrogen was added on 13 May; the grass tops were cut on 27 May and again on 10 June and 14 July. At the end of the experiment (14 July), the grass was clipped down to the soil surface in order to include the stubbles in the harvested material. Nitrogen balances were established at the end of the experiment as described in chapter II.C.1.

An *additional experiment* was carried out on sandy soil at 80 per cent of the waterholding capacity to investigate the influence of the cutting time on ^{15}N-balance and ^{15}N-distribution. The grass in this experiment was cut on the following dates:

Series I:	27 May	10 June	14 July
Series II:		10 June	14 July
Series III:		19 June	14 July

As series I the sods at 80 per cent W.H.C. of the preceding experiment were used. Series II consisted of 5 pots which were dressed with nitrate similarly to Series I. Series III consisted of 10 pots and was a combination of two planned series, each dressed with nitrate similarly to series II.

C. Results and discussion

1. *Temperature during the experiment*

No significant differences in temperature were observed between the three soil types and the lawn. The average maximum and minimum temperatures during the first twenty days of the experiment were 16.9 and 12.7°C respectively at −10 cm, and 21.6 and 8.1°C at +10 cm.

2. *Differences in dry-matter production and nitrogen ($^{14}+^{15}N$) yield between pots and rings*

The dry matter and nitrogen yields are presented as mean values of three and five replicates, respectively, in Table 4. Since no significant differences

TABLE 4. Dry-matter production (g per pot or ring) and nitrogen yield (mg $^{14}+^{15}N$ per pot or ring) of the herbage on sandy soil of pots and rings.

	Added NO_3–N mg per pot	Cutting date						Total		N-effect		
		14/7		10/6		27/5					N yield	
		Dry matter	N yield	Dry matter	N yield	Dry matter	N yield	Dry matter	N yield	Dry matter	mg	%
Pots	240	1.83	92.3	2.37	75.4	5.45	63.4	9.65	231.1	5.87	159.7	66
	0	0.68	16.5	0.33	8.0	2.77	46.9	3.78	71.4			
Rings	240	1.52	79.7	1.93	62.0	2.80	68.0	6.25	199.7	2.27	96.7	40
	0	1.11	28.9	0.37	10.6	2.50	63.5	3.98	103.0			

were observed between the values of the two moisture levels (80 and 95% W.H.C.) in the pots with sandy-soil sods, both series were averaged. The effect of added nitrate on dry-matter production and nitrogen yield (N-effect), estimated by subtracting the yields of the controls from those dressed with nitrate, was considerably higher in the pots than in the field. The low recovery of the fertilizer nitrogen under field conditions may have been due to uptake by the roots of plants grown outside the rings which had not been dressed with nitrogen. More soil was at the disposal of the plants in the field than in the pots, resulting in a higher uptake of soil nitrogen and consequently in a higher dry-matter production and nitrogen yield of the untreated rings.

3. *Effect of the moisture level on dry-matter production and nitrogen ($^{14}+^{15}N$) yield of the herbage*

The moisture level appeared to have no significant influence on either dry-matter production or nitrogen yield of the grass grown on clay and sandy soil. On peat soil, however, a pronounced effect was observed (Table 5). Except

TABLE 5. Effect of 240 mg nitrate nitrogen on dry-matter production (g per pot) and nitrogen ($^{14}+^{15}N$) yield (mg N per pot) of the herbage of sods derived from peat, clay and sandy soil.

	W.H.C. %	Treatment				Nitrogen effect		
		240 mg NO_3–N		No Nitrogen		Dry matter	N-yield	
		Dry[1] matter	N[1] yield	Dry[2] matter	N[2] yield		Dry matter	Percentage
Peat soil	80	7.93	193 ± 8	2.86	46 ± 6	5.07	147	61
	95	7.65	163 ± 1	3.41	45 ± 1	4.24	118	49
Clay soil	80	8.83	188 ± 6	3.01	35 ± 5	5.82	153	64
	95	8.19	181 ± 6	3.12	42 ± 13	5.07	139	58
Sandy soil	80	9.64	222 ± 7	3.91	75 ± 2	5.73	147	61
	95	9.65	238 ± 4	3.95	68 ± 12	5.70	170	71

[1] Mean values of 3 pots.
[2] Mean values of 2 pots.

in the case of peat soil at 95 per cent of the waterholding capacity (where a considerably lower value was found), the average figure for nitrogen recovery found in this experiment was about 65 per cent. Deviation from this value may have been due to variations between the sods, which is shown by the high standard deviations of the nitrogen yields.

4. *Nitrogen (^{15}N) balances at the end of the experiment*

The distribution of labeled nitrogen among herbage, roots and soil in the separate pots of each soil is presented in Table 6. The nitrogen balances of

TABLE 6. Distribution of labeled nitrogen among herbage, roots and soil at the end of the experiment.

Soil	Pot No	W.H.C. %	Mg ^{15}N applied	Herbage mg ^{15}N	Roots mg ^{15}N	Soil mg ^{15}N	Total mg ^{15}N	Not accounted for	
								mg ^{15}N	Percentage
Peat	8	95	80.0	33.3	1.5	12.7	47.5	32.5	41
	13	80	80.0	45.6	1.7	17.4	64.7	15.3	19
Clay	8	95	50.0	27.5	4.1	2.9	34.5	15.5	31
	12	95	50.0	29.9	5.6	6.5	42.0	8.0	16
	13[1]	80	50.0	25.7	6.5	1.5	33.8	16.2	32
	14	80	50.0	31.0	6.0	3.7	40.6	9.4	19
Sandy soil	20	95	50.0	30.8	4.0	2.8	37.7	12.3	25
	29	95	50.0	33.6	5.1	6.0	44.5	5.5	11
	23[2]	80	50.0	31.7	4.7	6.4	42.8	7.2	14
	26[2]	80	50.0	33.3	5.3	3.7	42.4	7.6	15
	25[2]	80	50.0	29.8	8.3	4.2	42.3	7.7	15
	24[2]	80	50.0	27.8	7.3	6.3	41.5	8.5	17
	22[2]	80	50.0	27.3	7.3	6.2	40.8	9.2	19

[1] Erroneously 480 mg N applied.
[2] Pots of the cutting-frequency experiment; 23 and 26, Series I (cut on 27 May, 10 June and 14 July); 25, Series II (cut on 10 June and 14 July); 24 and 22, Series III (cut on 19 June and 14 July).

all pots showed considerable deficits. These losses undoubtedly were due to volatilization. As to the mechanism of the latter process, the results of this experiment give no information. Volatilization of nitrogen from living plants has been claimed by PEARSALL and BILLIMORIA (83). Other investigators (2, 3), however, were unable to confirm these results. Therefore, losses by volatilization of nitrate nitrogen (denitrification) are more likely to have occurred. In two of the three soils tested, the influence of the moisture level on loss of nitrogen was not significant. Only in peat soil had volatilization increased at the high moisture level (Table 6). The low nitrogen yield in the herbage on this soil at 95 per cent of the waterholding capacity (Table 5) is in agreement with this result. In the clay and sandy soils, the differences between the separate pots do not allow any conclusion.

As mentioned in chapter II.B.6, the digestion method with H_2SO_4 and H_2O_2, used in this experiment to estimate the nitrogen content of the herbage, did not account for all of the nitrate the grass may have contained. Therefore, the real losses may have been some 5 per cent lower.

A linear negative correlation existed in the separate pots between the up-

take of fertilizer nitrogen by the herbage and the nitrogen not accounted for (Fig. 1). The consequence of this relationship is that, under field conditions, apart from leaching, the losses by volatilization determine to a high degree the recovery of fertilizer nitrate in the herbage.

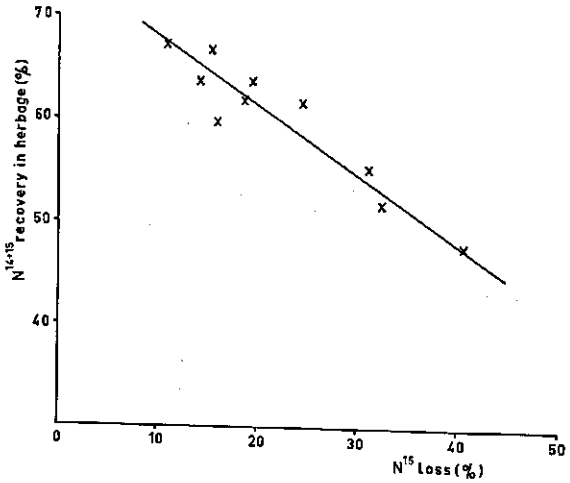


FIG. 1. Relationship between the nitrogen recovery in the herbage of the separate pots and the fertilizer nitrogen not accounted for.

At the end of the experiment the soil still contained considerable amounts of labeled nitrogen. As all mineral nitrogen had disappeared, it must be assumed that it had been incorporated into soil organic matter. In explaining the mechanism of this incorporation, the possibilities summed up in chapter I.B.2. had to be considered. The analysis applied to the experiments of WALKER (chapter I.B.2) could not be used in this experiment, as the ^{14}N-content of the herbage and roots at the beginning of the experiment had not been estimated. Therefore, it was impossible to decide if the ^{15}N-content of the soil-organic matter represented a net increase in soil nitrogen or merely was due to nitrogen turnover. Nor was it possible to establish whether or not part of the label found in the soil at the end of the experiment had passed the plant.

5. *The effect of cutting frequency on dry-matter production and nitrogen ($^{14}+^{15}N$) yield of the herbage*

Nitrogen yields and dry-matter production of the herbage at different cutting frequencies are presented in Table 7. The dry-matter production was considerably enlarged by delaying the first cut. This confirms the well-known fact that dry-weight production is reduced by higher cutting frequency. The nitrogen yield of the fertilized pots of series II and III was lower than that of series I. As the nitrogen yields of the unfertilized pots showed the same trend, the nitrogen effect was not significantly affected by the cutting frequency.

6. *The effect of cutting frequency on ^{15}N-distribution*

The influence of the cutting date on fertilizer-nitrogen distribution is shown in Table 6 (pots 23, 26, 25, 24, 22). The quantity of nitrogen not accounted

TABLE 7. Effect of cutting frequency on dry-matter production (g per pot) and nitrogen ($^{14}+^{15}N$) yield (mg N per pot) of herbage on sandy-soil sods.

NO_3–N mg per pot	Series	Cutting date								Total		Nitrogen effect		
		27 May		10 June		19 June		14 July					N-yield	
		Dry matter	N yield	Dry matter	N yield	Dry matter	N yield	Dry matter	N yield	Dry matter	N yield	Dry matter	mg	%
240	I[1]	1.92	98.1	2.12	71.9			5.19	58.4	9.23	228.4 ± 6.0	5.21	154.8 ± 10.0	65
0	I[2]	1.08	25.6	0.27	6.5			2.66	41.5	4.01	73.6 ± 6.1			
240	II[1]			5.16	137.2			6.13	66.2	11.29	203.4 ± 4.2	7.86	161.0 ± 8.0	67
0	II[2]			1.14	15.7			2.29	26.7	3.43	42.4 ± 6.1			
240	III[3]					7.61	136.1	6.22	63.4	13.83	199.5 ± 4.0	9.79	148.5 ± 4.0	62
0	III[4]					1.84	22.4	2.20	28.6	4.04	51.0 ± 1.2			

Averages of 3[1], 2[2], 6[3] or 4[4] pots.

TABLE 8. Distribution of total and fertilizer nitrogen (mg N per pot) in the herbage at different cutting times.

Series	Cutting date								Total		Indirect recovery I	Direct recovery D	D/I × 100%
	27 May		10 June		19 June		14 July						
	$^{14}+^{15}N$	^{15}N	$^{14}+^{15}N$	^{15}N	$^{14}+^{15}N$	^{15}N	$^{14}+^{15}N$	^{15}N	$^{14}+^{15}N$	^{15}N			
I 240 mg NO_3–N	98	68	72	55			58	32	228	155[1]	154	155	100
no nitrogen	26		6				42		74				
II 240 mg NO_3–N			137	112			66	31	203	143[2]	161	143	89
no nitrogen			16				26		42				
III 240 mg NO_3–N					136	94	63	38	200	132[3]	149	132	91
no nitrogen					22		29		51				

Mean values of 4[1], 1[2] and 2[3] pots.

for seems to have been unchanged by the cutting frequency. The amount of labeled nitrogen present in the roots at the end of the experiment, however, was considerably higher in the case of series II and III. This may be explained by assuming an increased uptake of fertilizer nitrogen by the herbage at a higher cutting frequency. A transport of labeled nitrogen from the herbage to the roots in the case of a delayed first cut is also possible.

At the end of the experiment, the amount of fertilizer nitrogen in the soil was somewhat higher in series III (Table 6); nevertheless the differences were not significant. An enlarged quantity of fertilizer nitrogen in the soil might indicate that after a delayed first cut, nitrogenous compounds had been excreted by the plants to the soil.

An analysis of the ^{14}N: ^{15}N distribution in the different cuts provides some indication concerning the replacement of fertilizer nitrogen by unlabeled nitrogen (Table 8). The effect of the fertilizer can be estimated in the conventional way by subtracting the nitrogen yields of the controls from those of the fertilized pots (Indirect Method). This method is based on the assumption that the uptake and the distribution of nitrogen among roots and herbage is the same in the treated and untreated plants. This is not entirely true. The fertilizer effect also can be determined directly from ^{15}N-data (Direct Method). The difference between both methods provides a measure of the biological exchange between ^{14}N and ^{15}N. In Table 8 the values for Direct Recovery have been calculated as percentages of those of Indirect Recovery. These values are lower with reduced cutting frequency. It is unlikely that the nitrogen turnover in the soil has been influenced by the cutting frequency. The only other explanation is, that in series II and III exchange between ^{14}N and ^{15}N has occurred inside the plants. This implies that part of the fertilizer nitrogen present in the tops has been transported downwards and replaced by ^{14}N. The downward movement can also been shown by comparing the first cuts of series II and III (Table 8). Although between 10 and 19 June the total nitrogen level of the herbage remained constant, the quantity of fertilizer nitrogen had considerably decreased (112 and 94 mg N, respectively). From these results it may be concluded that the Direct Method is unreliable for ascertaining the effect of fertilizer nitrogen.

D. Summary

Labeled nitrate was added to permanent grassland sods derived from peat, clay and sandy soil. The sods were maintained at 80 and 95 per cent of the waterholding capacity, field conditions being imitated as closely as possible. At the end of the experiment, nitrogen balances were established by estimating ^{15}N-content of herbage, roots and soil. Considerable losses by volatilization occurred (summarized data in Table 9), no influence of the moisture level

Table 9. Distribution of fertilizer nitrogen after addition of labeled nitrate.

Soil type	Herbage	Roots	Soil	Not accounted for
Sandy soil	65%	10%	10%	15%
Clay	57%	11%	7%	25%
Peat, 80% W.H.C.	57%	2%	22%	19%
Peat, 95% W.H.C.	44%	2%	17%	37%

being observed on clay and sandy soil (Table 6). A linear, negative correlationship was shown to exist between fertilizer uptake by the herbage and the quantity of nitrogen lost by volatilization. At the end of the experiment all soils still contained part of the fertilizer label in the organic-matter fraction. The experiment provided no information concerning the mechanism of this incorporation.

By varying the cutting frequency of the herbage with sandy-soil sods, it was shown that, by delaying the first cut, the roots retained more fertilizer nitrogen. There were indications that a downward movement of fertilizer nitrogen from the herbage to the roots and soil may occur (summarized data in Table 10).

TABLE 10. Influence of cutting frequency on fertilizer nitrogen distribution.

Cutting frequency	Herbage	Roots	Soil	Not accounted for
3 cuttings	65%	10%	10%	15%
2 cuttings	55%	14.5%	12.5%	18%

The information and hypothesis derived from these preliminary experiments were investigated further in separate experiments which are described in the next chapters.

CHAPTER IV

VOLATILIZATION, AND INCORPORATION OF FERTILIZER NITROGEN INTO SOIL ORGANIC MATTER

A. INTRODUCTION

In the preceding chapter it was shown that considerable amounts of fertilizer nitrogen were lost by *volatilization,* when nitrate was added to permanent-grassland sods. The mechanism of this volatilization, however, remained obscure. Losses from the grass plants as well as from the soil had to be considered. In order to decide which was the more likely possibility, nitrogen balances were established as soon as the nitrate had disappeared from the soil (T_0). A comparison was made with nitrogen balances of sods which were analysed one month later (T_1). A difference between these values would point to losses from the grass plants.

In order to investigate the *direct surplus incorporation* of fertilizer nitrogen into soil organic matter (see page 8), a further experiment with grass sods was carried out in which the living roots had been killed so that uptake by the plant was eliminated. Direct surplus incorporation of fertilizer nitrogen would result in a decrease of the mineral-nitrogen content of the soil.

B. EXPERIMENTAL PROCEDURE

The *volatilization experiment*[1] was performed in 1959 (under experimental conditions similar to those of 1958) in two series of sods derived from sandy soil. One series received 206 mg

[1] Experiment in collaboration with Ir. K. DILZ.

nitrate nitrogen (as a KNO_3-solution) of which 47 mg were labeled; the other series received 205 mg ammonium nitrogen (applied as a $(NH_4)_2SO_4$-solution), containing 50.2 mg ^{15}N. Each series consisted of 6 pots. Since the moisture content had not significantly influenced the losses in the previous year, the experiment was carried out at 60 per cent of the waterholding capacity. In two additional series of sods which had received the same quantities of nitrogen in the unlabeled form, the mineral nitrogen was estimated periodically in order to trap T_0. Three pots of each series with labeled nitrogen were analysed at T_0. The balances of the remaining pots were established at T_1. The nitrogen was added on 10 June. Sods of the nitrate series were analysed on 20 June (T_0) and 20 July (T_1); those of the ammonium-treated sods on 24 June (T_0) and 24 July (T_1).

The *direct surplus incorporation* of fertilizer nitrogen was ascertained by investigating the mineralization-immobilization turnover in sods with killed plants in three experiments, carried out at 60 per cent of the waterholding capacity. Sods from sandy soil, 11 cm in diameter and 7 cm thick, were incubated in the dark at 28°C after clipping the grass. In a second series the soil was thoroughly mixed. The subsoil (between –7 and –15 cm), which contained few roots, was also incubated. The mineral nitrogen content of each series was periodically estimated.

In a second experiment, sods 8 cm in diameter and 6 cm thick, contained in pots, were dug in to the level of the lawn. The grass was clipped several times to kill the roots and the soil surface was covered with Enkalon-fibre to prevent evaporation and growth of algae. When the roots had died, 20 pots received 100 mg ammonium nitrogen (as $(NH_4)_2SO_4$), 20 more pots were given 95 mg nitrate nitrogen (as KNO_3), and 20 pots were left without added nitrogen. The mineral-nitrogen content of the sods was estimated at different times in 4 replicate pots of each series.

The experiment was repeated with labeled ammonium and nitrate nitrogen, and was started at the same time as the volatilization experiment described above. Three pots received 200 mg nitrate nitrogen of which 30 mg were labeled; 4 pots received 200 mg ammonium nitrogen of which 28 mg were labeled. One of the ammonium-treated pots was analysed 1.5 hours after the nitrogen addition to estimate the chemical fixation of this compound. The remaining pots were analysed after 27 days.

C. Results and discussion

1. *Volatilization of fertilizer nitrogen*

The results of the volatilization experiment with labeled ammonium and nitrate nitrogen, applied to permanent grassland sods, are presented in Table 11. Climatological conditions prevailing during the experiment differed con-

Table 11. Nitrogen distribution at two cutting times upon addition of labeled ammonium and nitrate nitrogen to sods from permanent grassland.

Nitrogen applied (mg N per pot) and time of analysis	Mg ^{15}N	Pot no	Date of analysis	Herbage		Roots		Soil		Total		Not accounted for	
				mg ^{15}N	%	mg ^{15}N	%	mg ^{15}N	%	mg ^{15}N	%	mg ^{15}N	%
205, NH_4–N T_0	50.19	1	24/6	27.70	55.2	8.36	16.6	10.92	21.7	46.98	93.5	3.21	6.5
	50.19	3	24/6	26.47	53.1	8.93	17.7	9.96	19.5	45.36	90.3	4.83	9.7
	50.19	49	24/6	27.63	55.1	7.91	15.7	12.18	24.2	47.72	94.0	2.47	5.0
205, NH_4–N T_1	50.19	21	24/7	27.52	55.0	8.95	17.8	10.35	20.6	46.82	93.4	3.37	6.6
	50.19	24	24/7	27.67	55.2	8.70	17.3	9.40	18.8	45.77	91.3	4.42	8.7
	50.19	26	24/7	27.34	54.5	8.85	17.6	8.85	17.9	45.04	90.0	5.15	10.0
206, NO_3–N T_0	47.00	7	20/6	17.60	37.4	5.33	11.4	14.75	31.4	37.68	80.2	9.32	19.8
	47.00	12	20/6	18.85	40.1	4.70	10.1	12.49	26.6	36.04	76.8	10.96	23.2
	47.00	19	20/6	19.88	42.3	4.02	8.6	14.38	30.6	38.24	81.5	8.76	18.5
206, NO_3–N T_1	47.00	27	20/7	24.32	51.7	7.72	16.4	7.40	15.6	39.44	83.7	7.54	16.3
	47.00	28	20/7	28.27	60.1	8.53	18.2	7.85	16.7	44.65	95.0	2.35	5.0
	47.00	29	20/7	23.54	50.0	7.62	16.2	7.76	16.5	38.92	82.7	8.08	17.3

siderably from those during the experiment described in chapter III.C.4, when temperatures were considerably lower. The maximum and minimum temperatures during the first 15 days of the experiment were 25.5°C and 15.8°C, respectively, at –10 cm, and 29.4°C and 7.4°C, respectively, at + 10 cm.

In each of the pots the nitrogen was only partly recovered. The highest losses were observed with nitrate. The higher values at T_0, as compared with those at T_1, can be explained only by the variability of the sods. Since no losses occurred between T_0 and T_1, it is obvious that the deficits were caused by volatilization of mineral nitrogen from the soil and not by volatilization from the plants. It may be assumed that, in the case of nitrate, the losses were caused by denitrification, although this was not directly proved by gas analysis. An alternative explanation of the losses would be a microbial reduction to nitrite, followed by a chemical decomposition of this compound. Since all other results obtained in further experiments pointed to denitrification, and no indications were obtained in favour of the chemical decomposition of nitrite, the losses were ascribed to the former mechanism.

In spite of the low moisture level (60 per cent of the waterholding capacity), the losses after a nitrate dressing were higher than in the 1958-experiment. Since other experimental conditions were the same, this increase can be explained only by considering the abnormal high soil temperatures in 1959. Oxygen consumption by roots and microorganisms is stimulated by rising temperatures, while oxygen solubility is reduced. Both factors lower the quantity of oxygen dissolved in the soil solution, thus promoting denitrification which as such is also stimulated by the higher temperature. Therefore it is plausible that, in permanent-grassland soils, temperature is an important factor concerning losses caused by denitrification.

The mechanism causing the losses of nitrogen after an ammonium dressing remained unclear. Although some information was obtained in favour of nitrification, followed by denitrification, no serious attempt was made to clarify the process.

The nitrogen distribution among herbage, roots and soil was the same at T_0 and T_1 upon application of ammonium sulphate. In the case of nitrate treatment, however, a considerable part of the nitrogen, present in the soil at T_0, was transported afterwards to the plants. The fact, that at T_0 the soil contained higher amounts of labeled nitrogen when nitrate was used as a fertilizer than at T_1, cannot be explained in terms of an enlarged turnover, since microorganisms seem to prefer ammonium in the immobilization of mineral nitrogen (54).

2. *Incorporation of fertilizer nitrogen (^{14}N) into soil-organic matter of sods with killed roots: mineralization-immobilization experiments*

The mineralization rate of undisturbed and mixed sods without added fertilizer upon incubation at 28°C is shown in Table 12. In undisturbed sods, the amount of ammonium and nitrate nitrogen present was relatively low during the first 50 days of the experiment. This may have been caused by the presence of an excess of carbogeneous material which favours re-immobilization. In addition, the mineralization rate may have been slow. By mixing the sods, the rate of production of mineral nitrogen was very much enlarged. Obviously, carbogeneous material did not become available at a sufficient rate to re-immobilize the vigorously mineralized nitrogen. This different behaviour of

TABLE 12. Mineral-nitrogen content (ppm) of undisturbed sods and mixed sods (plants killed) and subsoil, upon incubation at 28 °C.

	pH	Incubation time (days)									
		0		8		26		53		112	
		NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-
Sods, undisturbed	5.3	0	0	0	19	0	16	0	27	78	125
Sods, mixed	5.3	0	0	4	28	8	63	9	132	34	244
Subsoil	5.4	0	0	0	2	0	26	0	52	0	92

undisturbed sods and mixed sods supports the hypothesis proposed in chapter I.B.1. that the high organic matter content of permanent-grassland soils in the temperate humid regions is caused by a slow decomposition of organic matter.

The experiment also indicated that the nitrification in the sods seems to be retarded compared with the subsoil.

In order to establish whether a surplus immobilization of added fertilizer nitrogen might occur, unlabeled ammonium and nitrate nitrogen were supplied to undisturbed sods with killed roots. The results of this experiment are recorded in Table 13. No surplus immobilization occurred with either ammo-

TABLE 13. Mineral-nitrogen content (ppm) of undisturbed sods with killed root systems, upon an addition of ammonium and nitrate nitrogen.

Nitrogen addition	Incubation time (days)									
	0		4		10		27		65	
	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-
100 mg NH_4–N	99.6	7.7	98.1	9.7	89.1	16.5	85.0	23.6	67.2	73.6
95 mg NO_3–N	19.0	92.1	20.6	86.8	23.1	86.7	29.1	85.3	24.1	71.8
No nitrogen	14.0	11.2	13.4	9.3	12.9	8.0	14.0	18.9	8.0	12.4

nium or nitrate nitrogen, since no decrease in the total level of mineral nitrogen was observed during the experiment. Therefore it is unlikely that this sandy soil has increased in organic nitrogen by direct immobilization of added fertilizer nitrogen under the influence of carbogeneous material present in the soil outside the living plants. Although it is possible that in sods with living roots, substances which may serve as a carbon cource are excreted by the plants, it is more likely that other mechanisms are involved in the accumulation of organic nitrogen in permanent-grassland soils.

During the first 27 days of the experiment the nitrate content was more or less constant (Table 13). This observation may indicate that during that period no denitrification occurred.

The mineral-nitrogen content of the sods which received no nitrogenous fertilizer was low during the entire experimental period. Since it was shown in this experiment that a surplus of carbogeneous material was not available (added fertilizer was not immobilized), it is obvious that the mineralization rate in undisturbed sods of this sandy soil is low.

3. *Volatilization, and incorporation of labeled fertilizer nitrogen into soil organic matter (sods with killed roots)*

The experiment described above was repeated with labelled nitrate and

ammonium nitrogen, in order to establish the differences between sods with living roots (see Table 11) and those with killed roots regarding volatilization and immobilization. The nitrogen fractions of sods with killed roots after addition of labeled fertilizer nitrogen, are recorded in Table 14. Compared

TABLE 14. Nitrogen distribution in sods with killed roots after an addition of labeled nitrate and ammonium nitrogen.

N-dressing	Mg ^{15}N	Pot no	Time (days)	Nitrate		Ammonium		Org. + fixed N		Total		Loss	
				mg ^{15}N	%	mg ^{15}N	%	mg ^{15}N	%	mg ^{15}N	%	mg ^{15}N	%
200 mg NO_3–N	30.00	66	27	25.95	86.4	0.04	0.1	0.89	3.0	26.88	89.6	3.22	10.4
	30.00	67	27	25.58	83.8	0.05	0.2	0.65	2.2	26.28	86.2	4.72	13.8
	30.00	70	27	24.84	82.9	0.03	0.1	4.17	13.8	29.05	97.0	0.95	3.0
200 mg NH_4–N	28.00	76	1.5h	0.28	1.0	25.81	92.2	2.71	9.6	28.80	102.8	+0.80	+2.8
	28.00	77	27	6.65	23.7	14.32	51.2	5.24	18.7	26.21	93.6	1.79	6.4
	28.00	79	27	13.34	47.7	9.85	35.2	4.73	16.9	27.92	99.7	0.08	0.3
	28.00	83	27	9.46	33.8	13.02	46.5	6.70	23.8	29.18	104.0	+1.18	+4.0

with the balances of sods containing living roots (Table 11), the losses were much lower. With ammonium sulphate the nitrogen was completely recovered, and in the case of nitrate the average loss was about 9 per cent versus 17 per cent in the system with living plants (Table 11). When the experiment was repeated in 1960, losses of about 3 per cent were observed, 19 and 51 days, respectively after the addition of nitrate. These observations can be interpreted only by assuming that the root system of living plants exerts a stimulating influence on the losses caused by denitrification. To explain this effect of living plants on denitrification, two mechanisms have to be considered. The oxygen uptake by roots and by rhizosphere organisms may reduce the quantity of oxygen dissolved in the soil solution, thus causing the conditions of oxygen deficiency needed in denitrification. Again the plants may stimulate denitrification by the excretion of organic compounds which can be used as hydrogen donors. The former certainly will be encountered, since no denitrification occurs in the presence of sufficient oxygen.

Except in the case of pot 70, only a small amount of the label present in the added nitrate was recovered in the soil organic matter (Table 14). When the experiment was repeated, the organic matter in the case of nitrate contained 6 per cent of the label and with ammonia approximately 20 per cent. This observation is in agreement with that of JANSSON (54), who explained the results of his incubation experiments by assuming that heterotrophic soil microorganisms prefer ammonia to nitrate as a nitrogen source.

The validity of this interpretation was shown by the present author in a number of experiments with pure cultures of soil microorganisms; the results of such an experiment, performed with an *Arthrobacter*-species, isolated from grassland soil, are shown in Table 15. The organism, which was able to use ammonium as well as nitrate as the sole nitrogen source, was grown in shaking cultures using a medium of the following composition: glucose, 4 g; yeast extract 0.1 g; casamino acids, 1 g; KH_2PO_4, 3.75 g; Na_2HPO_4, 4.8 g; minor elements; distilled water, 1 litre. The results of this experiment show that practically no nitrate was consumed as long as ammonium nitrogen was available.

TABLE 15. Ammonium and nitrate utilization by *Arthrobacter* str. 51.

Initial amounts mg N per l		Nitrogen recovered (mg N per l) after different periods of growth (days)							
		1.5		2.5		4		8	
NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-
110	304	47	303	35	305	24	304	27	304
84	340	3	339	2	346	2	341	6	340
41	402	3	386	3	386	6	384	4	388
16	456	8	437	4	436	4	435	4	438

A considerably higher percentage of the label from nitrate was found in the soil organic matter in sods with living roots, both at T_0 and T_1 (Table 11), than in sods with killed roots. The origin of this high level can only be ascribed to mechanisms in which the living plants are involved. Since denitrification is stimulated by the plants, nitrate may have been reduced to the ammonium level, followed by incorporation of the ammonium into soil organic matter. An alternative explanation is that part of the labeled nitrate taken up by the plants had been excreted to the soil.

In the case of ammonium fertilizer after 30 days, about 20 per cent of the nitrogen added to pots with killed roots was found in the organic matter and the clay minerals; about half of this quantity may have been fixed chemically (pot 76, Table 14). Since the total amount of mineral nitrogen ($^{14}+^{15}N$) had not decreased at the end of the experiment, no increase of soil-organic nitrogen had occurred; the increase of ^{15}N in soil organic matter (Table 14) has been the result of turnover. In the experiment with living roots (Table 11) the soil organic matter and clay minerals contained the same percentage of label as in the experiment with killed roots. Since it is not likely that living roots will repress mineralization, it must be assumed that the incorporation of fertilizer ammonia in the experiment with living roots (Table 11) will also have been the result of a turnover mechanism, not being attended with increases in organic soil nitrogen.

D. SUMMARY

By establishing nitrogen balances of permanent grassland sods at different times after the application of labeled nitrate and ammonia, it could be shown that part of the fertilizer was lost by volatilization of mineral nitrogen. In the case of nitrate, the losses were ascribed to denitrification. When the roots, present in the sods, were killed, the losses were considerably lower (Table 16). It was concluded that living plants have a stimulative effect on denitrification.

TABLE 16. The influence of living roots on nitrogen distribution and volatilization.

	N-source	Percentage label			
		Herbage	Roots	Fixed N + Org. N	Loss
Living roots	NO_3	50	17	16	17
	NH_4	55	17.5	19	8.5
		Mineral nitrogen			
Killed roots	NO_3	85		6	9
	NH_4	80		20	0

In the experiments using sods with killed roots, the mineralization rate appeared to be low and nitrification was retarded. Although in the sandy soil used in this investigation a considerable amount of ^{15}N derived from added fertilizer nitrogen was found to be incorporated in the soil organic matter, particularly in sods with living roots, no gains in soil nitrogen by surplus immobilization of fertilizer nitrogen were observed. Nitrate was involved only to a small extent in the nitrogen turnover. Indications were obtained that, with nitrate being used as a fertilizer, part of the nitrogen is incorporated in soil organic matter by mechanisms other than nitrogen turnover.

CHAPTER V

THE INFLUENCE OF LIVING PLANTS ON DENITRIFICATION, AND ON INCORPORATION OF FERTILIZER NITROGEN INTO SOIL ORGANIC MATTER: EXPERIMENTS WITH SODS AND SOWN GRASS

A. Introduction

By comparing nitrogen balances of nitrate-dressed sods with living and killed root systems, it was shown in the preceding chapter that living roots exert a stimulative effect on denitrification. This effect may be brought about by lowering the quantity of dissolved oxygen, excretion of organic compounds (hydrogen donors), or a combination of both. If this hypothesis is valid, then the large quantity of organic matter present in permanent-grassland sods is not an intrinsic factor in denitrification. The same effect would be observed in any soil with living roots, assuming that the quantity of hydrogen donors is not a limiting factor. To test this hypothesis, a comparison was made of nitrogen balances of permanent-grassland sods and sown grass after a nitrate dressing.

Also in the preceding chapter, strong evidence was obtained that part of the fertilizer nitrogen may be incorporated in soil organic matter by mechanisms in which the living plants are involved. Thus, nitrogen originally taken up by the plants is supposed to reach the soil in the form of excreted products or dead roots. In addition, these products may be used as hydrogen donors by denitrifying organisms. This point was also investigated by establishing nitrogen balances of sown grass.

B. Experimental procedures

In order to compare nitrogen balances of established sods and sown grass, an experiment[1] was designed, similar to that of the previous year with permanent-grassland sods. Labeled ammonium and nitrate nitrogen were added to two series of sods from sandy soil at 60 per cent of the waterholding capacity. Nitrogen balances were established in three sods of each series at T_0 and T_1. T_0 was estimated in two additional series with unlabeled nitrogen. The ammonium series were given 200 mg N of which 48.5 mg were labeled .The nitrate series were given 185.2 mg N of which 50 mg were labeled. The nitrogen was added on 4 June; nitrogen balances were established in both series on 23 June (T_0) and 25 July (T_1).

[1] Experiments of this chapter in collaboration with Ir. K. Dilz.

The experiment with sown grass was carried out in glass jars containing 1.5 kg sandy soil (pH 5.5) at 60 per cent of the waterholding capacity. To each of the jars, 0.6 g superphosphate and 0.6 g of a mixture of potassium and magnesium sulphates were added. Perennial ryegrass (containing 19 mg N) was sown on 21 May. The jars were arranged in three series according to the following scheme:

	Cutting date and date of analysis of herbage	Date of analysis of roots and soil
Series I	21 June	21 June
Series II	21 June, 25 July	25 July
Series III	25 July	25 July

Each series consisted of 8 pots, which on 21 May received the following treatments:

2 pots: 200 mg N, applied as a KNO_3-solution; herbage and roots analysed,
2 pots: 200 mg N, applied as a KNO_3-solution; herbage and total soil (roots + soil) analysed,
2 pots: 180 mg ^{14}N + 20 mg ^{15}N, applied as a KNO_3-solution; herbage and roots analysed,
2 pots: 170 mg ^{14}N + 30 mg ^{15}N, applied as a KNO_3-solution; herbage and total soil analysed.

Two additional series (Series IV and V) received 200 mg unlabeled nitrate nitrogen on 21 May. These series were cut on 25 July and subsequently received the following treatments:

Series IV:

2 pots: 171.3 mg ^{14}N + 28.7 mg ^{15}N, applied as a $(NH_4)_2SO_4$-solution; herbage and total soil analysed,
2 pots: 180 mg ^{14}N + 20 mg ^{15}N, applied as a $(NH_4)_2SO_4$-solution; herbage and roots analysed.

Series V:

2 pots: 170 mg ^{14}N + 30 mg ^{15}N, applied as a KNO_3-solution; herbage and total soil analysed,
2 pots: 180 mg ^{14}N + 20 mg ^{15}N, applied as a KNO_3-solution; herbage and roots analysed. Both series were analysed on 24 August.

In a second experiment with sown perennial ryegrass growing in Mitscherlich pots, containing 5.5 kg sandy soil (pH 5.5), unlabeled nitrogen was added. The experiment consisted of 3 series, each of 4 pots, receiving the following treatments: Series A, 600 mg N; Series B, 1200 mg N; Series C, 1800 mg N. The nitrogen was added on 2 April as ammonium nitrate limestone, simultaneously with 3 g superphosphate and 3 g of a mixture of potassium and magnesium sulphates. In two pots of each series, herbage and roots were analysed on 25 May, and in the remaining pots on 17 June.

C. Results and discussion

1. *Experiments with sods*

The soil temperatures during the experiment were intermediate between those of the previous years. The nitrogen balances of this experiment which are recorded in Table 17, are somewhat different from those obtained in 1959 (Table 11). Although part of the nitrogen had volatilized, the losses with nitrate were generally considerably lower and those with ammonia higher than in the previous year. With nitrate, especially at T_1, large differences were observed between replicate sods. The average losses after an ammonium dressing amounted to 10 per cent, after a nitrate dressing to 14 per cent.

At T_1 the roots contained considerably higher quantities of label than at T_0. Obviously large amounts of nitrogen were transported from the herbage to the roots during the ageing of the grass. The higher percentage ^{15}N-excess in the herbage at T_0 indicates that, afterwards, part of the labeled nitrogen in the tops was replaced by unlabeled nitrogen, thus suggesting an internal nitrogen cycle.

TABLE 17. Nitrogen distribution at two cutting times upon addition of labeled ammonium and nitrate nitrogen to sods from permanent grassland.

Mg N applied per pot, and time of analysis	Mg ^{15}N	Pot no	Date of analysis	Herbage			Roots		Soil		Total		Loss	
				mg ^{15}N	%	% ^{15}N excess	mg ^{15}N	%	mg ^{15}N	%	mg ^{15}N	%	mg ^{15}N	%
200 mg NH_4–N T_0	48.5	1	23/6	26.68	55.0	16.0	7.92	16.3	10.86	22.4	45.46	93.7	3.04	6.3
	48.5	15	23/6	27.55	56.8	14.6	6.38	13.2	8.20	16.9	42.13	86.9	6.37	13.1
	48.5	19	23/6	27.78	57.3	15.3	6.81	14.0	9.19	18.9	43.78	90.2	4.72	9.8
200 mg NH_4–N T_1	48.5	8	25/7	20.24	41.7	13.9	11.27	23.2	12.05	24.8	43.56	89.7	4.94	10.3
	48.5	10	25/7	18.47	38.1	14.5	12.95	26.7	10.67	22.0	42.09	86.8	6.41	13.2
	48.5	14	25/7	20.90	43.1	14.1	12.89	26.6	9.89	20.4	43.68	90.1	4.82	9.9
185.2 mg NO_3–N T_0	50.0	6	23/6	28.72	57.4	16.2	4.77	9.5	11.32	22.6	44.81	89.6	5.19	10.4
	50.0	20	23/6	32.34	64.7	15.8	6.87	13.7	8.00	16.0	47.21	94.4	2.79	5.6
	50.0	24	23/6	30.57	61.1	16.5	5.77	11.5	9.64	19.3	45.98	91.9	4.01	8.1
185.2 mg NO_3–N T_1	50.0	25	25/7	19.92	39.8	14.4	12.29	24.6	5.93	11.9	38.14	76.2	11.86	23.8
	50.0	26	25/7	24.37	48.7	16.7	11.93	23.9	10.56	21.1	46.86	93.7	3.14	6.3
	50.0	27	25/7	17.88	35.7	14.7	8.50	17.0	7.58	15.2	33.96	67.9	16.04	33.1

TABLE 18. Nitrogen distribution (mg N per pot) among herbage, roots and soil of sown perennial ryegrass at different stages of development, upon application of 200 mg partially labeled nitrate nitrogen.

Series and cutting date	Date of analysis	Herbage			Roots		Total plant		Soil		Total pot			Loss	
		$^{14}+^{15}N$	Fert. N	% ^{15}N excess	$^{14}+^{15}N$	Fert. N	$^{14}+^{15}N$	Fert. N	$^{14}+^{15}N$	Fert. N	$^{14}+^{15}N$	Fert. N	% ^{15}N	Fert. N	% ^{15}N
I 21/6	21/6	160.0 ±3.0	131.2 ±2.0	12.3[1] 8.2[2]	39.5 ±2.0	24.8 ±2.0	199.5 ±5.0	156.0 ±3.0	980.0 ±3.0	44.0 ±2.0	1180.0 ±3.0	200 ±3	100	0	0
II 21/6, 25/7	25/7	171.0 ±3.0	137.0 ±2.0	12.3[1] 8.3[2]	41.9 ±2.0	24.8 ±1.0	212.9 ±3.0	162.0 ±3.0	965.0 ±4.0	24.0 ±4.0	1177.0 ±4.0	186 ±4	93	14	7
III 25/7	25/7	130.0 ±1.0	99.0 ±1.0	11.3[1] 7.5[2]	74.6 ±2.0	53.0 ±2.0	204.6 ±3.0	152.0 ±2.0	975.0 ±4.0	32.0 ±3.0	1180.0 ±4.0	184 ±4	92	16	8

[1] 30 mg ^{15}N applied; [2] 20 mg ^{15}N applied.

With ammonia, the soil also contained more of the label at T_1 than at T_0; the differences were, however, hardly significant. These results were in agreement with those obtained in the cutting-time experiment (Table 6). In both experiments part of the nitrogen, originally present in the herbage, was transported to the roots during the ageing of the plants, and, to a smaller extent, to the soil.

2. *Experiments with sown grass*

The nitrogen balances of the pots of Series I, II and III with sown grass are presented in Table 18. Since different amounts of labeled nitrogen were applied, the ^{15}N-values are presented as fertilizer nitrogen; the fertilizer nitrogen was calculated by multiplying the ^{15}N-values with a factor 10 in the case of 20 mg ^{15}N being applied, and with 6.66 in the case of 30 mg ^{15}N. The nitrogen content of the soil was estimated by subtracting the nitrogen content of the roots from the total amount of soil nitrogen (roots + soil).

The results of this experiment show that, exept in the case of Series I, significant nitrogen losses occurred. Unfortunately Series I was analysed before the fertilizer nitrogen was completely taken up by the plants. Therefore, this series cannot be compared with Series II and III. The losses were smaller than those with sods of permanent grassland of the same pH. With Series IV and V, in which labeled ammonium and nitrate nitrogen were added to plants which had been cut once, the same results were obtained. In the case of nitrate, 8.5 per cent and in that of ammonium nitrogen 9.3 per cent of the label were not recovered. In an additional experiment potassium nitrate and ammonium sulphate were added to the same soil without plants. After 3 months incubation at 28°C, the added nitrogen of both sources was completely recovered. Therefore, it is obvious that in the experiment with sown grass, the living plants also have stimulated denitrification.

Recently a similar experiment was carried out by BROADBENT and TYLOR (20). These investigators arrived at the opposite result, *viz.* higher losses of nitrogen in unplanted soils than in soils planted with Sudan grass, with both nitrate and ammonium nitrogen. Since these investigations were performed with alkaline soils, volatilization of ammonia may have occurred which may have been prevented by uptake by the plants. In the case of nitrate, one of the other factors (described in chapter I.C. of this thesis) which may cause denitrification, will have reduced the quantity of dissolved oxygen, since also in the absence of plants losses occurred. Under such conditions the plants will prevent the complete denitrification of the added nitrate by uptake of part of the dressing. Therefore, a promoting effect of living plants on denitrification may only be expected, when no other factor is lowering the quantity of dissolved oxygen below the critical level.

During the ageing of the grass plants, considerable amounts of nitrogen were transported to the roots (Table 18). By clipping the tops (Series II), the downward movement of the nitrogen was repressed. At the end of the experiment, total fertilizer nitrogen taken up by the plants was 162 mg N in Series II and 152 mg N in Series III. This indicates that, during the ageing of the grass of Series III, part of the nitrogen (approximately 10 mg N) was transported to the soil. Incorporated fertilizer nitrogen of the soil in Series III was indeed higher than that in Series II (32 *viz.* 24 mg N).

From the data presented in Table 18, it is possible to calculate whether gains in organic soil nitrogen had occurred. The difference between $^{14}+^{15}N$

and fertilizer-nitrogen content of the plants was 53 mg N in Series III. The nitrogen content of the seeds was 19 mg N. Consequently 34 mg of mineralized soil nitrogen had been taken up by the plants at the end of the experiment. At the same time 32 mg of the fertilizer nitrogen were incorporated into soil-organic matter. Therefore, it is obvious that in Series III no significant gains or losses of organic soil nitrogen had occurred. This result is in disagreement with that of the experiments of WALKER (119), described in chapter I.B.2.a,

TABLE 19. Nitrogen content of herbage and roots (mg N per pot) of perennial ryegrass, sown in Mitscherlich pots, at different stages of development.

Added N mg per pot	Date of analysis						Transported to the soil (by difference of row 4 and 7)
	25 May			17 June			
	Herbage	Roots	Total	Herbage	Roots	Total	
600 mg N	398	256	654	336	236	572	82
1200 mg N	813	378	1191	702	369	1071	120
1800 mg N	1201	451	1652	1088	484	1572	80

from which it could be calculated that significant increases in organic soil nitrogen had occurred when nitrogen was added to sown perennial ryegrass.

The experiment with sown grass was preceded by an identical one with unlabeled nitrogen (ammonium nitrate limestone) in Mitscherlich pots. In this experiment considerable quantities of nitrogen were transported to the soil as is shown in Table 19. On 25 May the highest amount of nitrogen applied was not completely taken up by the plants. The discrepancy in nitrogen transport to the soil between the glass jars and the Mitscherlich pots was doubtlessly caused by the size of the vessels. When the experiments were repeated, also with unlabeled nitrogen, the same results were obtained. Again considerable quantities of nitrogen were transported to the soil during the ageing of plants grown in Mitscherlich pots, while in glass jars the nitrogen content of the plants remained nearly constant. With plants grown in culture solution, no decreases of the nitrogen content were observed (DILZ, unpublished data). Although the experiments described in this chapter clearly demonstrate that nitrogen may be transported from the plants to the soil, the conditions under which the process takes place, are still rather obscure. As to the mechanism of the transport, the experiments give no decisive answer. Excretion of nitrogenous compounds, as well as breakdown of dying roots, may be responsible for the phenomenon. Transport to the soil may also occur during the earlier stages of the development of the plants. JANSSON (55) has shown that remineralization of labeled nitrogen, which was immobilized during nitrogen turnover, starts after only 6 days. Uptake from this source will obscure an eventual transport from the plants to the soil, since the quantity of label measured in the soil is the overall result of both processes. The high amount of label found at T_0 in organic soil nitrogen, when a nitrate dressing was given to permanent-grassland sods (Table 11, 17), may have been caused by excretion of nitrogenous compounds during the earlier stages of development of the plants.

D. Discussion of the experiments described in chapters III, IV and V

The principal aim of the experiments, described in the preceding chapters, was to trace the fate of fertilizer-nitrogen dressings applied on permanent grassland. Leaching, denitrification of nitrate nitrogen and immobilization in soil organic matter were expected to play an important role in preventing the nitrogen from being taken up by the plants. Under varying experimental conditions where no leaching was possible, 45–65 per cent of a nitrate dressing was found in the herbage, 10–25 per cent in the roots, and 7–22 per cent in the soil, while 10–40 per cent was lost by denitrification. When ammonium nitrogen was supplied, about 10 per cent was lost by a volatilization mechanism.

It appeared that denitrification was stimulated considerably by the presence of living plants. The results obtained with sown grass suggest that denitrification may occur in any soil with living roots; in arable soils as well as in permanent grassland.

In the sandy soil used in the present investigation, no direct immobilization of fertilizer nitrogen in soil organic matter occurred other than by the normal turnover mechanism. After an ammonium dressing, 10 per cent of the label was fixed chemically in clay minerals or organic matter of the soil. The situation may be different in other soils, especially in peat soils.

During the ageing of the grass, considerable amounts of fertilizer nitrogen may return to the soil. The mechanism of this process remained unclear. The experiments gave no proof that the process would result in an increase of soil nitrogen, since at the same time mineralized nitrogen was taken up by the plants, completing a turnover mechanism in which the plants are involved. The transport of organic nitrogenous compounds to the soil may start at an early stage of development of plants, thus supplying hydrogen donors to denitrifying organisms. In the short-time experiments, described in this thesis, increases in soil nitrogen by roots dying during the winter and by excrements of cattle were not considered.

The total quantity of nitrogen present in herbage and roots when all mineral nitrogen is taken up from the soil (N_t), is determined by the quantity of fertilizer nitrogen (F), the quantity of mineralized nitrogen (M), the initial nitrogen content of the plants or seeds (I), the quantity of nitrogen lost by leaching (L), and the losses by volatilization (V), or $N_t = F + M + I - L - V$. The validity of this equation was tested in some pot experiments with sown grass. Two of these tests will be discussed here.

Glass jars containing sandy soil and sown perennial ryegrass were supplied with a solution of ammonium nitrate in amounts of 100, 200 and 300 mg nitrogen per pot, respectively. The seeds contained 15 mg N. When the nitrogen had been taken up, the plants were analysed. The total amounts of nitrogen in the plants were 130, 213 and 301 mg nitrogen per pot, respectively. The assumption was made that the mineralization of soil nitrogen was not influenced by the addition of fertilizer nitrogen and that a constant percentage V of the fertilizer volatilized. The following equations were derived:

$$\text{Series I} : 130 = 100 + 15 + M - 100V \quad (1)$$
$$\text{Series II} : 213 = 200 + 15 + M - 200V \quad (2)$$
$$\text{Series III}: 301 = 300 + 15 + M - 300V \quad (3)$$

From these equations M and V may be solved. The following M-values were obtained:

M = 32 (equations 1 and 2 combined)
M = 23 (equations 2 and 3 combined)
M = 29 (equations 1 and 3 combined)

In a series of control pots to which no nitrogen was added, a M-value of 28 mg was obtained, thus showing the validity of the assumptions. When the known M-value of 28 mg was substituted in the equations, the losses appeared to be 13–14 per cent, which demonstrated that a constant percentage was volatilized. Both mineralization and denitrification were found to be strongly stimulated, when the experiment was repeated at a higher soil temperature.

As a second example, the results of Table 18, Series II were used. In this series the plants contained 212.9 — 162.0 = 51 mg non-fertilizer nitrogen, of which 32 mg were derived from the soil and 19 mg from the seed. The soil contained 24 mg fertilizer nitrogen. Thus the net mineralization was 32 — 24 = 8 mg N. Therefore, the equation in this example will be: 213 = 200 + 19 + 8 — V. From this equation a V-value of 14 mg was obtained, which agrees with the value obtained by substituting the ^{15}N-values of herbage (H), roots (R) and soil (S) in the formula F = H + R + S + V, as was done in the experiments described in the preceding chapters.

The equation described above enables the study of the effect of environmental conditions on denitrification and mineralization without using labeled nitrogen, by analysing total nitrogen content at two fertilizer levels.

E. Summary

In chapter IV strong evidence was obtained concerning a promoting effect of living plants on denitrification. By comparing nitrogen balances of permanent grassland with those of grass sown in pots, the validity of this assumption was made probable.

In both the grass sods and the sown grass, a mechanism was shown to be operating by which, during the ageing of the plants, nitrogen was transported from the tops to the roots and in some cases also to the soil. The existence of a nitrogen turnover mechanism, in which the plants are involved, was made probable.

The results presented in the chapters III, IV and V were briefly discussed and recapitulated. From the results an equation was derived which enabled the study of denitrification without making use of labeled nitrogen.

CHAPTER VI

FACTORS EFFECTING DENITRIFICATION IN THE RHIZOSPHERE

A. Introduction

In the preceding chapters it was shown that living plants stimulate denitrification in soil. This effect might be due to two factors. Firstly low oxygen tensions might occur in the rhizosphere, either caused by respiration of the root system, or by activities of the microflora using root excretions as sub-

strates for aerobic respiration. Secondly, root excretions might serve as a source of hydrogen donors during denitrification.

Experiments pertaining to the influence of these factors on denitrification are described below. In studies of this type, other factors which also effect denitrification (e.g. oxygen deficiency caused by high moisture content of the soil, effect of soil organic matter) have to be eliminated. For that reason several of the experiments were carried out with a simplified soil-plant system in which perennial ryegrass *(Lolium perenne)* and green pea *(Pisum sativum)* were sown in vermiculite, free from organic matter.

B. Consumption of oxygen in the rhizosphere

1. *The root-soil system as a whole*

To gain an impression of the contribution of living roots to the total oxygen consumption of permanent grassland, the uptake of sods with a living root system was compared with that of sods with killed roots. Small sods, 4.5 cm in diameter and 3 cm thick, at 55 per cent of the waterholding capacity, were placed in Warburg vessels of 130 ml capacity, containing 4 ml of a 4 per cent KOH-solution for the absorption of carbon dioxide. The oxygen uptake was measured at 25°C.

The uptake by sods with killed roots appeared to be 0.9 μl O_2 per gram of dry soil per hour, whereas in the presence of living roots 25.1 μl O_2 per gram of dry soil per hour was consumed (mean values of 5 replicates). These data support the hypothesis that in soils containing living roots, low oxygen tensions are more commonly encountered than in fallow land of the same soil type.

2. *The components of the root-soil system*

The increased oxygen consumption by sods containing living roots will have been the result of uptake by roots and rhizosphere organisms. It is a well-known fact that the number of microorganisms in the rhizosphere is considerably higher than in the non-rhizosphere soil (106). In addition, it has been proved (59) that the rhizosphere organisms are more active in oxidyzing various substrates than non-rhizosphere organisms. It thus might be expected

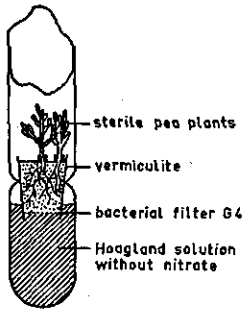


FIG. 2. Technique used for the cultivation of pea plants under sterile conditions.

that a considerable part of the observed oxygen consumption was due to microbial activities. To determine the extent of these activities, oxygen uptake of sterile and non-sterile root systems was measured. Since experiments with sterile grass plants are difficult to perform, the experiment was carried out with green peas.

Seeds of this plant species were sterilized with 3 per cent hydrogen-peroxide

solution. After germinating, two seeds were placed under sterile conditions in bacterial filters, containing vermiculite (Fig. 2). The latter was freed from organic matter, and brought to pH 6.8 after three subsequent washings with Hoagland solution. The tubes containing the sterile peas were placed in a light cabinet under controlled growth conditions. When 3 days old, one series of peas was inoculated with 1 ml of a soil suspension; a second series was kept sterile. A bacterial filter containing vermiculite inoculated with soil suspension but without pea plants served as a blanc. After 14 days, the filters, after removal of the tops of the plants, were placed in sterile Warburg vessels; subsequently the oxygen uptake of both series was estimated. At the end of the Warburg experiment sterility was controlled by plating the vermiculite of the sterile tubes on casein-soil extract-agar.

An oxygen consumption of 3.2 ml per gram of dry roots per hour was measured in the case of sterile pea roots; 4.8 ml oxygen was taken up by non-sterile roots. No oxygen was consumed by the unplanted filters, indicating that the vermiculite contained no organic substrates to be used by microorganisms. When the experiment was repeated, again 67 per cent of the total oxygen consumption was accounted for by the roots, the remainder (33 per cent) by the microorganisms. This ratio of 2 : 1 agrees with that found by LUNDEGÅRDH (69) for carbon dioxide production by sterile and non-sterile root systems of various plant species.

3. *Discussion*

It is obvious that in soils containing living roots the oxygen consumption is not evenly distributed in the soil, but is mainly restricted to the root region. Therefore, the conditions in the immediate root environment determine the occurrence of oxygen deficiency to a high degree. Although it was incidentally proposed (81) that anaerobic conditions could temporarily occur in the rhizosphere, no direct proof was obtained of a higher denitrification rate in the rhizosphere than in the non-rhizosphere soil, and in 1958 STARKEY (106) stated: "There is no evidence that denitrification is rapid in the rhizosphere, although it may be more rapid than in soil owing to the greater microbial activity which might provide local areas of anaerobiosis." The data presented in the preceding chapters show, however, that denitrification is considerably higher in the presence of living plants.

The occurrence of anaerobic conditions in the rhizosphere was also demonstrated in soil physical investigations by WIEGAND and LEMON (121). These workers found that oxygen diffusion to the root surface depends to a high degree on the diffusion rate through the water film surrounding the roots. It was calculated and afterwards confirmed in experiments that in many soils the oxygen concentration on the root surface is the limiting factor for plant growth and that this concentration sometimes will drop to zero. However, in the calculations of WIEGAND and LEMON only the oxygen consumption by higher plants was considered, that of rhizosphere organisms was not taken into account. Therefore, it may be concluded that oxygen deficiency in the root region will occur easier than was assumed by these investigators and that consequently the conditions required for denitrification will often occur. For that reason it is assumed that the effect of living plants on denitrification in soil has to be ascribed in the first place to bringing about oxygen tensions which permit denitrification in the rhizosphere.

C. Hydrogen donors supplied by plants

1. *Introductory remarks*

In addition to creating anaerobic conditions in the rhizosphere, living plants may promote denitrification by root excretions serving as a source of hydrogen donors for denitrifying organisms. With respect to this it should be mentioned that perennial ryegrass under certain conditions may supply considerably amounts of nitrogenous compounds to the soil (chapter V). However, the results recorded in that chapter provided no indications concerning the nature of these compounds.

Although a considerable number of investigations has dealt with the excretion of organic compounds by roots, the quantitative aspects of the process have been hardly investigated. Rovira (95) surveyed in a recent publication the compounds, excreted by plants. A large number of products, among which amino acids and sugars were the most abundant, was detected in root exudates. Since paper chromatography and microbial-assay methods were generally used to trace these compounds, most reports give only qualitative estimates. The few papers in which quantitative data are presented (93, 94), deal with investigations using sterile plants. It is possible, however, that under non-sterile conditions the continuous removal of the excreted compounds by the microorganisms on the root surface promotes the excretion process. Under such conditions the excreted products will immediately be converted by the microorganisms to cell material, so that the detection of the compounds themselves is prevented. Therefore, it is not surprising that it was recently found by Harmsen and Jager (48) that much higher quantities of organic compounds are excreted by non-sterile plants than is generally assumed.

In order to investigate whether higher plants excrete organic compounds at a rate which permits denitrification in the rhizosphere, perennial ryegrass was sown in vermiculite which contained no organic matter. In such a system only products, originating from plant roots, are available as a source of hydrogen donors during denitrification.

2. *Nitrogen balances of the plant-vermiculite system*

Perennial ryegrass seeds (containing 0.74 $\pm$ 0.04 mg N per basket) were sown in a series of plastic baskets, 4.5 cm in diameter and 3 cm high, filled with vermiculite at 60 per cent of the waterholding capacity. The vermiculite, previously freed from organic matter, was washed three times with Hoagland solution, and contained 3.48 $\pm$ 0.03 mg of nitrate nitrogen per basket. After germination, the seed was inoculated with 1 ml of a soil suspension containing 0.14 $\pm$ 0.01 mg N. An additional series of unplanted baskets was treated similarly. The grass was grown for a period of 30 days in a light cabinet at 20°C. The unplanted baskets were kept in the dark to prevent growth of algae, which were found to be able to supply organic compounds to other microorganisms. During this period, small amounts of nitrtate were periodically supplied to keep the grass growing; in total 10 mg of nitrate nitrogen were added. After 30 days, five baskets of each series were analysed. At this time the planted baskets contained 13.87 $\pm$ 0.13 mg N, and the unplanted ones 14.08 $\pm$ 0.07 mg N. Subsequently 10 mg of nitrate or 10 mg of ammonium nitrogen were added to both planted and unplanted baskets. After 45 days the nitrogen content of these baskets was estimated (Table 20). It will be seen that

TABLE 20. Nitrogen recovery of 10 mg of nitrate and ammonium nitrogen added to planted and unplanted baskets containing vermiculite free from organic matter.

Nitrogen added mg per basket	Nitrogen recovery			
	Planted		Unplanted	
	mg N	% of added N	mg N	% of added N
10 mg NO_3–N	21.5 ± 0.4	71.4	24.0 ± 0.3	99
10 mg NH_4–N	24.0 ± 0.8	100.0	23.9 ± 0.2	98
0	15.7 ± 0.2		14.1 ± 0.1	

a considerable percentage of the nitrate was volatilized in the planted baskets: the ammonium nitrogen, however, was completely recovered. Since no losses were observed in the unplanted baskets, denitrification can only have proceeded by the utilization of hydrogen donors derived from perennial ryegrass plants. From this experiment it appears that grass plants supply a sufficient amount of substrate to denitrifying organisms to permit denitrification.

3. *Experiments in Warburg vessels under anaerobic conditions*

Since the method used to study denitrification in soil by means of nitrogen balances was laborious, it was abandoned and replaced by the Warburg technique. If some precautions are taken, this technique proved to be very suitable for studying soil respiration; it appeared to be useful in denitrification studies as well. Recently the technique was adopted by MC GARITY (37) to investigate the effect of soil organic matter on denitrification.

To establish the effect of root excretions on denitrification, perennial ryegrass was grown in the same way as in the previous experiment. At different stages in the development of the plants, the tops were clipped 1 cm above the surface of the soil. The baskets were then placed in large Warburg vessels, containing 4 ml of a 4 per cent KOH-solution and 10 mg of nitrate nitrogen were added. Then the vessels were attached to the manometers; the air inside the vessels was replaced by nitrogen gas, which was freed from oxygen by passing it through a column containing heated, reduced metallic copper. Assuming that no products were evolved other than nitrogen gas, nitrous oxide and carbon dioxide, denitrification in the presence of grass plants of different ages could be followed manometrically (Fig. 3). It appeared that the quantity of hydrogen donors supplied by roots of perennial ryegrass, 4 days old, was insufficient to effect complete denitrification of the added nitrate. At 14 days, the plants apparently placed much more substrate at the disposal of the denitrifying organisms; denitrification continued until the nitrate was completely converted to gaseous products. The same denitrification rate was observed with perennial ryegrass at 28 days.

Since it was likely that the excretion of hydrogen donors was not restricted to perennial ryegrass, the experiment was repeated with root systems of green peas which were grown in the same way as those of perennial ryegrass. With this species a considerably higher denitrification rate occurred than with perennial ryegrass (Fig. 3).

To explain the diverse effect of root systems of peas and ryegrass on denitrification, it was supposed that the hydrogen donors excreted by peas were a more favourable substrate for denitrifying organisms. With respect to this,

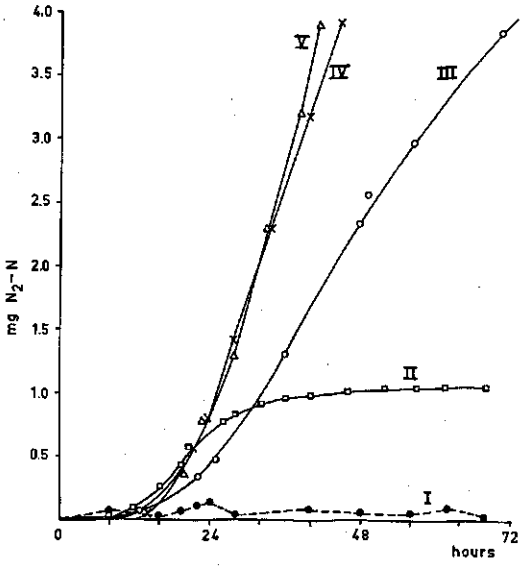


FIG. 3.
Formation of gaseous reduction products (expressed as mg N_2) during denitrification of 10 mg nitrate nitrogen with hydrogen donors supplied by root systems of perennial ryegrass and pea plants of different ages. I, unplanted control; II, perennial ryegrass, 4 days old; III, idem, 14 or 28 days old; IV, green peas, 8 days old; V, idem, 21 days old.

experiments of ROVIRA (93) should be mentioned, in which it was shown that sterile pea plants excreted more amino acids than sterile monocotyles. Since in the pot experiments with perennial ryegrass (chapter V) considerable amounts of nitrogenous products were found to be excreted, it seemed worthwile to investigate the effect of amino acids on denitrification in more detail. The experiments performed on the influence of amino acids on denitrification by pure cultures of denitrifying organisms will be recorded in the following chapters.

4. *Experiments in Warburg vessels under aerobic conditions*

The drawback in studying denitrification with the technique as used in the experiment described above is that the plants are placed under anaerobic conditions, so that they soon die. These dead plants may have induced enlarged excretion or decomposition. It appeared to be possible, however, to perform the same experiment under aerobic conditions, if the following principle was used.

The initial pressure in a Warburg vessel at the beginning of an aerobic experiment is equivalent to 1000 cm Brodie's solution, assuming an atmospheric pressure of 760 mm Hg. The partial oxygen pressure in the vessel is equivalent to 200 cm. When the oxygen in the vessel is completely consumed, an oxygen uptake equivalent to 200 cm can be read on the manometer, irrespective of the size of the vessel. However, air, equivalent to 200 cm must be introduced into the vessel during the experiment, in order to permit manometer readings at a constant volume. The partial oxygen pressure of the introduced air is equivalent to 40 cm. When the introduction of air is continued, the additional supply of the oxygen gradually decreases to zero. At the same time (T_0) an oxygen consumption corresponding to manometer readings which approach asymptotically to 250 cm (200 + 40 + 8 + 1.6 + 0.32) can be read on the manometer. This principle is based on the assumption that evolved carbon dioxide is taken up by alkali, and that no gasses, other than carbon

dioxide and oxygen, are produced or consumed. However, when gaseous nitrogen or nitrous oxide are given off during the experiment, due to denitrification, less than 250 cm will be read. The deviation from this value is a measure of the production of nitrogen gas and nitrous oxide. In that case the net gas consumption will change at T_0 into gas production.

Using this principle, an aerobic Warburg experiment was carried out under the same experimental conditions as in the previous experiment, in which 10 mg of nitrate nitrogen were added to root systems of pea plants (Fig. 4). When

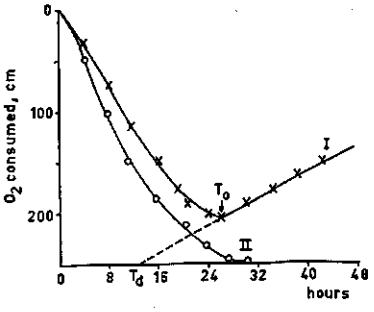


FIG. 4.
Pressure changes (in cm Brodie's solution) as caused by O_2-uptake by non-sterile root systems of pea plants (II, no nitrogen added), and O_2-uptake and gas production after the addition of nitrate to non-sterile plants (I).

T_0 was reached, an apparent oxygen uptake of 208 cm was measured (I). A series of pea plants, without added nitrate, had taken up oxygen, equivalent to 251 cm (II). It may be concluded that 251 — 208 = 43 cm nitrogen gas and nitrous oxide were evolved during the experiment. When it is assumed that the denitrification rate before and after T_0 was the same, the point T_d at which denitrification started, can be calculated (Fig. 4). This point appeared to be 13 hours after the experiment began. After 13 hours about half of the oxygen inside the vessel was consumed; under such conditions the plants will not have been damaged. The results consequently prove that living plants may supply sufficient hydrogen donors to the rhizosphere organisms to permit denitrification.

5. *Discussion*

From the experiments described in this section it appears that the conditions for denitrification are very favourable in the immediate environment of the roots. Oxygen deficiency is easily obtained, while sufficient hydrogen donors are available. Moreover, a continuous supply of nitrate is assured after the application of nitrate-containing fertilizers, as a consequence of the uptake by the roots, thus causing a constant flow of this soluble compound through the rhizosphere. Finally, the number of denitrifying organisms in the rhizosphere is much higher than that in non-rhizosphere soil. Therefore, it seems justified to conclude that in soils under vegetation, like permanent-grassland soils, denitrification will occur preferably in the vicinity of the roots.

D. SUMMARY

By comparing the oxygen uptake of permanent-grassland sods, containing living roots, with that of sods, containing killed roots, it was shown that living roots and rhizosphere organisms may considerably reduce the quantity of oxygen in the soil. The roots were found to consume 66 per cent of the total oxygen consumption of a non-sterile root system, the remainder being taken

up by the rhizosphere organisms. A sufficient quantity of hydrogen donors proved to be excreted by the roots of grass and pea plants to permit denitrification in the rhizosphere. Indications were obtained that different types of compounds were excreted by roots of perennial ryegrass and peas, the latter (possibly because of animo acids) causing a higher denitrification rate. From the results it was concluded that the rhizosphere constituted an excellent environment for the occurrence of the denitrification process.

CHAPTER VII

THE MICROORGANISMS OF GRASSLAND SOILS

A. INTRODUCTION

The operation of the nitrogen cycle in permanent grassland soils is largely the result of an interaction between microorganisms and higher plants, as was shown in the preceding chapters. For a further study of this interaction more knowledge was required of the microflora of these soils, particularly of the species involved in denitrification. Experiments pertaining to the microflora of grassland soils will be described below.

B. THE MICROFLORA OF GRASSLAND SOILS

1. *Literature*

Relatively few investigators have dealt with the microflora of permanent-grassland soils. WIERINGA (122) found that generally more than 50 per cent of the total number of microorganisms counted in pastures consisted of actinomycetes. Ploughing up the soil resulted in a rapid decrease in the number of actinomycetes with a concomitant rise in the number of bacteria. HARMSEN (45), studying aerobic cellulose decomposition in various soils, observed that in permanent-grassland soils mainly actinomycetes were involved in the process.

An extensive study of the microflora of New Zealand pastures was made by THORNTON and his collaborators (110), who showed that *Streptomyces*-species accounted for 41–58 per cent of the total population. It was suggested that the fungus flora in these soils represented a rhizosphere population. *Pseudomonas*-species were reported to be the most numerous bacteria in these soils.

2. *Methods*

The microflora of grassland soils was studied using the plating method. Although this method is subject to many difficulties (e.g. selectivity of the medium, influence of sampling, preparatory treatment) it can give satisfactory results, if a comparison is made of different soil samples treated in the same way.

From the grassland soils to be investigated 10 samples of each 100 g were taken. The combined samples were thoroughly mixed; then two aliquots of 5 g were suspended in 500 ml of a 0.8 per cent NaCl-solution. After shaking mechanically during 1 hour, two samples of 5 ml were taken from the suspension which was stirred during sampling. From each aliquot a dilution series was prepared. From the final dilution 5 samples of 0.5 ml were taken which were distributed with a bent glass rod over casein-agar plates (diam. 13 cm). The agar medium contained: casein, 5 g; K_2HPO_4, 1 g; agar 10 g; soil extract, 1 litre. The casein was added as a saturated solution in lime water. The agar plates were dried on a hot plate (50 °C) before use, so that water was readily taken up, thus preventing the spread of bacteria. The final dilutions were prepared so as to allow about 100 colonies to develop on each plate. The average number

of microorganisms on replicate plates was multiplied by the dilution; in this way the total number of microorganisms per gram oven-dry soil could be calculated. The moisture content of the initial soil sample was estimated separately.

3. *The population of established-grassland sods under field conditions*

a. Occurrence of actinomycetes, fungi and bacteria. The microflora of permanent-grassland sods derived from sandy soil which were used throughout this investigation, was studied under different climatological conditions. A comparison was made with two different unplanted arable soils. For that purpose the proportions of bacteria, actinomycetes and fungi were estimated with the plating method. The bacteria were subdivided into Gram-negative rods, bacilli and arthrobacters, by examining microscopically 100 strains from each sample. Arthrobacters were identified as organisms with rod form, sometimes branched cells in 18-hours old cultures on glucose-yeast extract-agar and coccoid forms on casein agar after 5 day's incubation at 28 °C.

The actinomycetes (mainly *Streptomyces*-species) were relatively more numerous in pasture than in arable soil (Table 21) which confirmed the results

TABLE 21. Population of permanent-grassland sods derived from sandy soil, under different climatological conditions, as compared with that of unplanted arable soil.

Soil conditions	% of W.H.C.	Total population $\times 10^6$/g	Percentage of total			Total bacteria $\times 10^6$/g	Percentage of bacteria		
			Bacteria	Actinom.	Fungi		G(-) rods	Bacilli	Arthrobacters
Pasture, winter	90	9.3	40	59	1	3.7	53	23	24
Pasture, spring	75	16.0	32	64	4	5.1	58	10	30
Pasture, summer	68	10.2	47	52	1	4.7	43	14	43
Pasture, summer	5	6.4	70	30	0	4.5	63	19	18
Arable soil, sand[1]	60	5.6	66	31	3	3.7	16	20	64
Arable soil, clay[1]	55	8.6	90	9	1	7.7	11	14	75

[1] Unpublished data of J. Antheunisse and E. G. Mulder.

of other investigators (45, 112). Of the bacteria, the percentage Gram-negative rods was found to be considerably higher in permanent-grassland sods than in arable soils, arthrobacters being relatively less numerous. The coccoid forms of the latter from permanent grassland were on the whole more refractive on microscopic examination.

b. The rhizosphere character of the bacterial population of grassland soils. The bacterial population of permanent-grassland soils – high percentages of Gram-negative rods, low percentages of arthrobacters (Table 21) – resembles the microflora usually found to be associated with the roots of graminaceous plants. In the rhizosphere of these plants, the number of Gram-negative rods in general is also relatively high, that of bacilli and arthrobacters being proportionally low (106, 42). As an exception SPERBER and ROVIRA (105), however, found a much higher proportion (78 per cent) of branched forms in the rhizosphere of grass plants.

In an investigation of the nutritional requirements of 140 bacteria from the counting plates, the resemblance between the bacterial population of grassland sods and that associated with grass roots was more definitely demonstrated (for the composition of the media, cf. chapter VIII). The results of this investigation (Table 22), showing the occurrence of a high proportion (about

TABLE 22. The nutritional requirements of bacteria isolated from a permanent-grassland soil.

Group of bacteria	Number of tested cultures	Simplest medium showing growth (% of total group)				Medium showing maximum growth (% of total group)				Growth stimulated by amino acids (%)
		c	cv	a	av	c	cv	a	av	
Gram-negative rods	80	54	0	44	2	0	0	98	2	100
Arthrobacters	51	41	41	6	12	29	29	13	29	42
Bacilli	9	0	0	89	11	0	0	89	11	100
Total collection	140	46	15	33	6	11	11	66	12	78

c Basal medium + ammonia
cv Basal medium + ammonia + B-vitamins
a Basal medium + amino acids
av Basal medium + amino acids + B-vitamins

80 per cent) of organisms (mainly Gram-negative rods) stimulated by amino acids, are similar to those obtained by GYLLENBERG (43) with strains isolated from the rhizosphere or graminaceous plants. It is a well-known fact (106) that also in the rhizosphere of other plant species the number of Gram-negative rods stimulated by amino acids, is much higher than in the non-rhizosphere soil.

4. *The effect of fertilizer nitrogen on the microflora of recently sown grassland soil*[1]

a. Experimental. Plots of sandy arable soil of different pH were partly sown with perennial ryegrass in the spring of 1960. The plots received different amounts of fertilizer nitrogen, applied as ammonium nitrate limestone. Samples from grassland plots at pH 6.5 without added nitrogen, from grassland plots with an annual dressing equivalent to 480 kg per ha (3 applications of 160 kg) and from the same plots cropped with arable crops (receiving 60 kg N annually), were taken periodically, and the number of bacteria, actinomycetes and fungi was estimated as described in VII.B.2. The bacteria were subdivided in Gram-negative rods, arthrobacters and bacilli.

b. Occurrence of bacteria, actionomycetes and fungi. It will be seen (Table 23) that as a result of the nitrogen application the total number of microorganisms in the grassland plots had increased already in the first year

TABLE 23. Number of microorganisms present in arable sandy soil and grassland (sown in the spring of 1960), after application of different amounts of fertilizer nitrogen.

Soil	Sampling date	Total number × 10^6/g	Bacteria		Actinomycetes		Fungi	
			Number × 10^6/g	%	Number × 10^6/g	%	Number × 10^6/g	%
Arable soil 60 kg N per ha anually	1-12-1960	3.5	2.3	66	1.1	31	0.1	3
	1-1-1962	3.2	1.9	61	1.2	31	0.1	2
	15-8-1962	2.6	1.6	65	0.9	33	0.1	2
Grasland no nitrogen	1-12-1960	4.2	2.5	60	1.6	38	0.1	2
	1-1-1962	2.4	1.5	62	0.8	36	0.1	2
	15-8-1962	2.1	1.5	66	0.5	33	0.1	1
Grassland 480 kg N per ha anually	1-12-1960	6.7	5.2	78	1.4	20	0.1	2
	1-1-1962	4.5	3.4	76	0.9	19	0.2	5
	15-8-1962	7.1	6.0	85	1.0	14	0.1	1

[1] Experiment carried out in collaboration with Ir. K. DILZ.

after sowing the grass. This increase was completely due to the higher numbers of bacteria. The numbers of actinomycetes and fungi had remained constant. It is remarkable that the number of actinomycetes, two years after the grass had been sown, did not increase as one would expect. No significant differences were observed between arable soil, cropped with various crops, and grassland soil that had received no fertilizer nitrogen.

c. Occurrence of Gram-negative rods, arthrobacters and bacilli. The favourable effect of fertilizer nitrogen on bacterial numbers was chiefly due to the response of Gram-negative rods and arthrobacters (the latter to a lesser extent). The bacilli were not affected by the fertilizer treatment (Table 24). Similar results were obtained with plots of different pH.

TABLE 24. Number of Gram-negative rods, bacilli and arthrobacters, present in arable soil and grassland (sown in the spring of 1960), after application of different amounts of fertilizer nitrogen.

Soil	Sampling date	Number of bacteria $\times 10^6$/g	Gram-negative rods		Bacilli		Arthrobacters	
			Number $\times 10^6$/g	%	Number $\times 10^6$/g	%	Number $\times 10^6$/g	%
Arable soil	1-12-1960	2.3	1.16	50	0.53	24	0.61	26
60 kg N per ha	1-1-1962	1.9	0.85	45	0.52	28	0.51	27
annually	15-8-1962	1.6	0.55	34	0.32	20	0.73	45
Grassland	1-12-1960	2.5	1.22	47	0.43	17	0.85	34
no nitrogen	1-1-1962	1.5	0.81	54	0.44	29	0.25	16
	15-8-1962	1.5	0.43	29	0.62	42	0.43	29
Grassland	1-12-1960	5.2	2.80	54	0.47	9	1.92	37
480 kg N per ha	1-1-1962	3.4	2.22	65	0.84	25	0.34	10
anually	15-8-1962	6.0	4.32	72	0.42	7	1.26	21

5. *Discussion*

It is a well-known fact that the root mass of permanent-grassland soils is mainly restricted to the top layers. Therefore, it was supposed that permanent-grassland sods obtained from the top layers would contain many organisms associated with plant roots. The results of this section which demonstrate a close relationship between the bacterial population of permanent-grassland soils and that generally found in the rhizosphere of grass plants are in agreement with this hypothesis. The situation in permanent grassland can be considered as a continuation of the process occurring in arable soils, where during the ageing of the crops the non-rhizosphere flora shows an increasing resemblance to the rhizosphere flora (44). Similar results were obtained with subantarctic soils by BUNT and ROVIRA (21). In these soils which are permanently covered by plants, no differences appeared to exist between the bacteria of the soil and those associated with plant roots.

In plots of arable soil sown with grass, a rapid increase in the number of Gram-negative rods occurred after application of fertilizer nitrogen (Table 24). The stimulative effect may have been the result of an enlarged root excretion, presumably of nitrogenous compounds, since in separate experiments (not recorded) no effect on bacterial numbers was observed when nitrogen was added to unplanted soils. A comparable effect on the number of Gram-negative rods was described by ROUATT and KATZNELSON (92), who observed

a rising number of bacteria requiring amino acids in the rhizosphere of plants grown at increasing light intensities.

In the present investigation no attempts were made to elucidate the function of the actinomycetes which always form an essential part of the microflora of established permanent-grassland soils. Since these organisms are known to utilize cellulose, lignin, and other insoluble compounds, all of which are difficult to attack, they may be involved in humus formation by decomposition of dead roots, from which the readily available compounds have been removed by the more rapidly growing bacteria and fungi.

A comparison of the data of Table 21 (permanent grassland, about 20 years old) and Tables 23 and 24 (sown grass, 0–2 years old) reveals that in the established sods actinomycetes were numerous, but that on the contrary no increase of these organisms had occurred in the sown grassland as compared with arable soil. In planted arable soils (Tables 23 and 24) more Gram-negative rods and less arthrobacters were present than in unplanted arable soils (Table 21) which demonstrates the stimulative effect of plants on the former organisms.

6. *Summary*

The microflora of permanent-grassland soils was investigated under different climatological conditions. Actinomycetes generally represented more than 50 per cent of the total population. The number of Gram-negative rods was found to be considerably higher and that of arthrobacters to be lower than in arable soils. It was concluded that the bacterial flora of permanent-grassland soils represents a rhizosphere population. An investigation of the nutritional requirements of these bacteria, of which about 80 per cent appeared to be stimulated by amino acids, underlined the rhizosphere character of the bacterial flora. Application of fertilizer nitrogen to grassland soils resulted in an increase of the number of Gram-negative rods which was in agreement with the above-mentioned conclusion.

C. The denitrifying bacteria of permanent-grassland soils

1. *Literature and introductory remarks*

After the first isolation of denitrifying bacteria by Gayon and Dupetit (38), a considerable number of different microorganisms was shown to possess a denitrifying capacity. Most of these organisms were isolated from enrichment cultures and it is unknown whether they are involved in the denitrification process in the soil. Beyerinck and Minkman (9) observed that during the first hours after inoculating enrichment media with soil, *Bacillus*-species were the most numerous. Thus, they supposed that these organisms were the most important ones in soil denitrification, a view which was endorsed by Verhoeven (115). However, the concept was never verified by isolating bacilli directly from soil upon the addition of nitrate.

A considerable number of investigations, reviewed by Starkey (106), has dealt with the occurrence of denitrifying bacteria in the rhizosphere of higher plants, where they were found to be present in much higher numbers than in the non-rhizosphere soil. This rise is almost certainly not due to the denitrifying capacity of the bacteria, since, unless nitrate has been added as a fertilizer, this compound will not be available in sufficient amounts in the rhizosphere, to effect a selection of denitrifying organisms. The similar behaviour

of denitrifying and ammonifying bacteria in the rhizosphere of mangels (58) suggests that the stimulation of the former may be the result of their ammonifying capacity.

As far as known to the present author, no investigations have dealt with the denitrifying organisms of permanent-grassland soils. The rhizosphere character of the bacterial population of these soils (see VII.B) warrants the supposition that they will occur in abundance. In the present investigation the denitrifying population of permanent-grassland soils was studied before and after the addition of nitrate. Experiments pertaining to this subject are recorded below.

2. *Methods*

a. Determination of denitrifying capacity. The denitrifying capacity of pure cultures of microorganisms was tested using a method described by VERHOEVEN (115). A few ml of a dense bacterial suspension in sterile water were prepared. One half of the suspension was mixed in a test tube with 20 ml of a melted-agar medium, consisting of: peptone, 10 g; glycerol, 10 g; potassium nitrate, 10 g; agar, 10 g; tap water, 1 litre; the medium was cooled to 45 °C. After mixing, the agar was solidified by further cooling. The other half of the suspension was mixed with an analogous medium without potassium nitrate. Those bacteria which showed good growth and gas formation in the nitrate-containing medium, but showed little or no growth and no gas formation in the medium without nitrate, were considered to possess a dissimilatory nitrate-reducing enzyme system. The method is recorded below as VERHOEVEN's method.

A number of strains which showed no denitrifying capacity, when tested using VERHOEVEN's method, was tested anew, using a modification of a medium described by SMITH *et al.* (103). It contained: tryptose, 10 g; beef extract, 3 g; yeast extract, 2 g; K_2HPO_4, 5 g; $NaNO_3$, 10 g; tapwater, 1 litre. Approximately 8 ml of this broth were put in 16 mm tubes and autoclaved. If the medium was not used immediately after preparation, it was steamed to drive off the free oxygen, then quickly cooled and supplied with 0.16 ml of a separately sterilized 50 per cent glycerol solution to make the final concentration 1 per cent. The tubes were then inoculated, capped with 15 mm of sterile, melted vaspar, and incubated at 30 °C. Tubes containing the same medium without nitrate were set up as a control. Gas production and growth were used as criteria for denitrifying capacity. The method is recorded below as SMITH's method.

b. Determination of the denitrifying population of established sods under field conditions without nitrate being added. The denitrifying capacity of 400 strains (70 *Streptomyces* and 330 bacterial strains) isolated from the sandy-soil pasture by the plate method (VII.B.2) was tested, using VERHOEVEN's method.

The total number of denitrifying organisms in this pasture was estimated by preparing a dilution series of a soil sample as described in VII.B.2. Of each dilution, 5 samples of 1 ml were mixed in test tubes with 20 ml of the peptone-glycerol-nitrate-agar medium described above (VII.C.2.a). After the agar had solidified, the tubes were incubated at 28 °C. The highest dilution showing gas production was used to estimate the total number of denitrifying organisms. From the tubes 40 strains were isolated which were tested using VERHOEVEN's method. Strains giving a negative result were tested again using SMITH's method. Positive strains were identified.

c. Determination of the denitrifying population of established sods upon the addition of nitrate. To each of 5 sandy-soil sods (at 55 per cent of the waterholding capacity), which were 11 cm in diameter and 7 cm thick, 2.1 g potassium nitrate were added, a treatment which was repeated twice at intervals of three days. Then the sods were incubated in a nitrogen atmosphere at 28 °C. After 10 days, a series of dilutions was prepared from a mixed sample of the 5 sods, as described above (VII.B.2). From the dilutions five samples of 0.5 ml were taken which were distributed with a sterile, bent glass rod over agar plates which had been dried previously. The agar medium contained: glycerol, 1 g; peptone, 1 g; yeast extract 0.1 g; KNO_3, 3 g; agar, 10 g; tap water, 1 litre. The agar plates were incubated at 28 °C. The dilution allowing about a hundred colonies to develop on the plates was used, to calculate the total number of microorganisms per gram oven-dry soil. The number of denitrifying organisms was estimated by testing 100 strains, isolated from the plates, using VERHOEVEN's method. Positive strains were identified.

The same estimations were performed with two series of sods without added nitrate which served as blancs. One of these series was incubated in a nitrogen atmosphere, the second aerobically, both at 28 °C.

3. *Results*

a. Denitrifying organisms of established sods under field conditions without nitrate being added. When 400 strains, isolated from permanent grassland, were examined for denitrifying capacity, using VERHOEVEN'S method, the tests with 70 *Streptomyces*-strains gave a negative result; of the remaining 330 bacterial strains, 35 appeared to possess a denitrifying capacity. Of these isolates, 33 were identified as *B. cereus* or *B. cereus* var. *mycoides*, one as *B. circulans* and one as a *Pseudomonas*-species. The tests with *Arthrobacter*-strains gave a negative outcome. Some further *B. cereus*-strains which also formed part of the collection of grassland microorganisms, tested for denitrification, were unable to denitrify.

The total number of denitrifying organisms of the grass sods, estimated by mixing dilutions of a soil suspension through a nitrate-containing agar medium, was approximately 3.10^6 organisms per gram oven-dry soil.

The identification of 40 strains, isolated from the tubes, gave the following result: *B. cereus* including var. *mycoides*, 24; *B. circulans*, 6; *B. macerans*, 3; *B. laterosporus*, 1; *B. coagulans*, 1; strongly refractive coccoid forms (unidentified), 5 strains. *Pseudomonas*-species and *B. licheniformis* were not found. When the strains were tested for denitrification, using Verhoeven's method, negative results were obtained, except with 21 of the *B. cereus* isolates. Since the negative results only demonstrated that the tested organisms did not exhibit nitrate-reducing activity in the medium adopted for the test, positive results might have been obtained with a different medium. Therefore, the strains were re-investigated using Smith's method. In this case all *Bacillus*-strains produced gas in the presence of nitrate, demonstrating the presence of a dissimilatory nitrate-reducing enzyme system in these organisms. The unidentified coccoid forms showed growth only in the presence of nitrate, but no gas was produced.

b. Denitrifying organisms of established sods after the addition of nitrate. The results of a comparison made between the denitrifying organisms of permanent-grassland sods, incubated with or without nitrate, are shown in Table 25. The denitrifying population of sods, incubated anaerobically

TABLE 25. Numbers of organisms, present in permanent-grassland sods, incubated with and without nitrate; numbers in 10^6 per gram oven-dry soil.

Microorganisms	Conditions of incubation		
	Anaerobic with nitrate	Anaerobic without nitrate	Aerobic without nitrate
Total number	85	8	32
Bacteria	85	8	24
Total denitrifying org.	73	6	4
Pseudomonas str. 241	39	0	0
Achromobacter str. 181	12	0	0
B. macerans	12	0	0
B. cereus	2	5	3
Other denitrifying org.	8	1	1
Other bacteria	12	2	20

with nitrate, consisted mainly of *Pseudomonas* str. 241, *Achromobacter* str. 181 and *B. macerans*, although small numbers of other denitrifying organisms were also present. Numbers of *B. cereus* had not increased and *B. licheniformis* was not isolated under these conditions. *B. cereus* was isolated as the predominant denitrifying species from sods incubated without nitrate. In the sods, incubated aerobically, the proportion of bacteria was high as compared with that of actinomycetes. In separate experiments this result was shown to be due to the selectivity of the agar medium which favoured the growth of bacteria more than did casein agar.

4. *Discussion*

A comparison of the denitrifying populations of grassland sods, without and with nitrate addition, revealed that under normal field conditions the population consisted mainly of *Bacillus*-species, of which *B. cereus* was by far the most numerous. Addition of nitrate resulted in the development of a flora of denitrifying bacteria which consisted mainly of *Pseudomonas, Achromobacter* and *B. macerans*-strains.

Since the denitrifying bacteria, found under normal field conditions, without nitrate addition, appeared to be not involved in the denitrification process, it is likely that their presence, at least in the sods tested in the present experiment, is not the result of their denitrifying capacity, but depends on some other characteristic. Using the same techniques, *B. cereus* was found to be also the most common denitrifying organism in two arable soils which had received no nitrate. The results obtained with permanent-grassland soil indicate that a direct relationship between the original number of denitrifying organisms and the denitrifying capacity of the soil, as was assumed to exist by POCHON (86) and VALERA and ALEXANDER (113), may not be expected, unless the soil has been treated regularly with nitrate.

Although *B. cereus* and some other *Bacillus*-species have been recorded to produce gas in the presence of nitrate (103), these organisms, except *B. licheniformis*, are generally not considered as denitrifying organisms. As early as 1910, BEYERINCK and MINKMAN (9) isolated denitrifying bacilli with not swollen and with definitely swollen sporangia from enrichment cultures. They proposed that these organisms belonged to one polymorphic species which they named *B. nitroxus*. VERHOEVEN (115), using enrichment media containing 8 per cent potassium nitrate, only isolated a denitrifying *Bacillus*-species with a not swollen sporangium, an organism which was identified as *B. licheniformis*. In the experiments described above, the original observations of Beyerinck and Minkman were found to be correct, since *B. circulans, B. macerans* and *B. laterosporus* are characterized by a definitely swollen sporangium. Recently, Dr. F. D. Cook, Ottawa, (personal communication) also observed the denitrifying capacity of *B. circulans;* the organism appeared to reduce nitrate mainly to nitrous oxide.

5. *Summary*

The denitrifying population of permanent-grassland soils was investigated without nitrate addition and with nitrate addition. Under natural conditions (without nitrate added) the population of organisms with denitrifying capacity consisted nearly exclusively of *Bacillus*-species of which *B. cereus* was the most numerous. In the denitrification process mainly *Pseudomonas, Achro-*

mobacter, and *B. macerans*-strains were involved, (sods incubated anaerobically in the presence of nitrate). It was concluded that the presence of denitrifying organisms under normal field conditions is not the result of their denitrifying capacity. Therefore, no direct relationship between the original denitrifying population and the denitrifying capacity of the soil may be expected.

D. Capacity of denitrifying organisms to utilize root excretions

1. *Introductory remarks*

In the preceding chapter it was shown that the conditions in the immediate root environment of grass plants (e.g. oxygen deficiency, root excretions, continuous supply of nitrate after a fertilizer dressing with this compound) were favourable for the occurrence of denitrification in permanent-grassland soils. During denitrification in these soils, *Pseudomonas* str. 241, *Achromobacter* str. 181 and *B. macerans* were the main representatives of the denitrifying population (VII.C). Since root excretions may be used as a source of hydrogen donors during denitrification (Fig. 3), the capacity of the three organisms to use these compounds during denitrification had to be investigated. With respect to this, a comparison was made with the denitrifying species which were found to occur under normal field conditions. Experiments on this subject are recorded below.

2. *Experimental*

a. Methods. Sterile pea plants were grown during 12 days in a vermiculite medium containing Hoagland solution, free of nitrate, as described in chapter VI.B. After the tops of the plants were cut, the bacterial filters containing the vermiculite medium with sterile root systems, were transferred to sterile Warburg vessels (containing 4 ml of a 4 per cent KOH-solution to absorb carbon dioxide). Suspensions of pure cultures of different denitrifying organisms were added along with a sterile solution containing 10 mg of nitrate nitrogen. After replacing the air inside the vessels by sterile nitrogen gas, the reduction of nitrate to gaseous products was estimated manometrically. Planted filters without pure cultures but with nitrate added and those with pure cultures but without nitrate added, served as blancs.

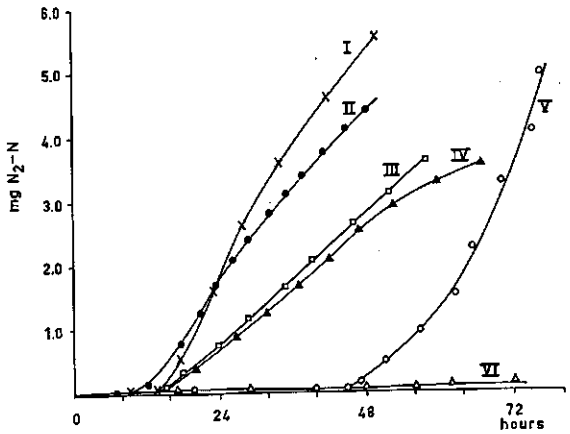
After 4 days the contents of the filters were transferred to 300 ml Erlenmeyer flasks containing 100 ml of a 1 N K_2SO_4-solution. The flasks were shaken mechanically during 1 hour. Then the content of the flasks was filtered, and the vermiculite on the filter was leached with K_2SO_4-solution until 250 ml of the filtrate were collected. Nitrate, nitrite and ammonium nitrogen were estimated in the filtrate as described in chapter 2.

b. Microorganisms. Pure cultures of denitrifying organisms isolated from grassland sods, with and without added nitrate, served as test organisms in these experiments. *Ps. aeruginosa*, *B. licheniformis*, *Sp. itersonii* and *Spirillum* str. 425, isolated from enrichment media inoculated with grassland soil, and *M. denitrificans* (obtained from the culture collection of the Laboratory of Microbiology, Wageningen) were also tested. The experiments were repeated several times and the results obtained showed a similar trend. Examples of typical experiments will be given below.

3. *Results and discussion*

a. Formation of gaseous reduction products. The rate of gas production from nitrate by a number of denitrifying species with root excretions supplied as the sole source of hydrogen donors, is recorded in Fig. 5. The species involved during denitrification in permanent-grassland soil (*Pseudomonas* str. 241, *Achromobacter* str. 181 and *B. macerans*) were all able to denitrify under the experimental conditions, while denitrifying species present under normal field conditions (*B. cereus*) were not. The latter was also true of *B. licheniformis*, and the two *Spirillum*-species. Gas evolution by *M. denitrificans* began only

FIG. 5.
Gaseous products (expressed as mg N_2-N) formed during the denitrification of 10 mg nitrate nitrogen by pure cultures of denitrifying organisms, using hydrogen donors, supplied by pea plants. I, *Pseudomonas* str. 241; II, *Ps. aeruginosa*; III, *Achromobacter* str. 181; IV, *B. macerans;* V. *M. denitrificans*; VI *B. cereus, B. licheniformis, Sp. itersonii, Spirillum* str. 425, blancs (root systems + nitrate, root systems + pure cultures).



after a prolonged lag period. The denitrification pattern of both *Pseudomonas*-strains had much in common with that of non-sterile pea plants (Fig. 3). The results support the hypothesis that *B. cereus* and *B. licheniformis* are of restricted importance in the denitrification process in the soil.

b. Formation of inorganic reduction products and nitrogen balances. The amounts of reduction products of nitrate, formed during denitrification by some of the tested species, are given in Table 26. With all species the added nitrogen was nearly completely recovered. *B. licheniformis*, although unable to reduce nitrate to gaseous compounds, produced considerable quantities of nitrite as well as some ammonia. The origin of the ammonium fraction was not clear. Formation by reduction of nitrate or by desamination of organic compounds are both possible.

TABLE 26. Products formed during the denitrification of 10 mg of nitrate nitrogen by pure cultures of denitrifying organisms, using hydrogen donors supplied by pea plants.

Species	Nitrogen recovered in mg N				
	NO_3^-	NO_2^-	NH_4^+	$N_2 + N_2O$	Total
Ps. aeruginosa[1]	3.75	2.40	0.52	3.04	9.71
Ps. aeruginosa	0.20	0.00	0.27	10.30	10.77
M. denitrificans	2.76	0.00	0.31	6.80	9.87
Sp. itersonii	9.00	0.45	0.14	0.00	9.59
B. licheniformis str. P 1	5.35	3.75	0.95	0.00	10.05
Sterile control	9.80	0.07	0.11	0.00	9.98

[1] Analysis after 2 days.

4. *Summary*

An investigation has been made of the capacity of denitrifying bacteria, isolated from permanent-grassland soil, to utilize root excretions as a source of hydrogen donors during denitrification. *Pseudomonas* str. 241, *Achromobacter* str. 181 and *B. macerans* which were mainly involved in the denitrification process in this soil were found to produce gas from nitrate. No gaseous reduction products were formed by *B. cereus* and *B. licheniformis*, species which were not involved in the denitrification process in this soil. A considerable quantity of nitrite was found to be produced by the latter species.

CHAPTER VIII

THE NUTRITIONAL REQUIREMENTS OF THE DENITRIFYING BACTERIA

A. INTRODUCTION

In the Warburg experiments described in chapter VI.C.3 (Fig. 3) a higher denitrification rate was observed when root excretions of non-sterile pea plants were used as the sole source of hydrogen donors instead of compounds excreted by perennial ryegrass. Since pea plants are known to excrete higher amounts of amino acids than monocotyles, the promoting effect of the former species on denitrification was supposed to be due to the excretion of these compounds. To prove the validity of this hypothesis, the effect of amino acids on denitrification by the microorganisms, responsible for the process in the soil, had to be investigated. The experiments described in the preceding chapter gave rise to the conclusion that *Pseudomonas* str. 241, *Achromobacter* str. 181 and *B. macerans* were the main representatives of the population involved in the denitrification process in grassland soils. These species were able to use root excretions of pea plants as a source of hydrogen donors during denitrification (chapter VII.D.3.a). The experiments pertaining to the effect of nutritional conditions (particularly amino acids) on growth and denitrification of these organisms are recorded below. In addition, a comparison was made between the nutritional requirements of these species and those of the bacteria with denitrifying capacity (mainly *Bacillus*-species) present in the soil under normal field conditions. The latter species proved to play no part in the denitrification process in the soil (Table 25), and they were unable to produce gas from nitrate when supplied with root excretions of pea plants as the sole source of hydrogen donors (Fig. 5). The comparison of the nutritional requirements might answer the question, why these organisms, although initially being more numerous, are outstriped by the species mentioned above after a nitrate addition to the soil.

The nutritional requirements of the denitrifying bacteria, mentioned in Bergey's Manual of Determinative Bacteriology, are more or less known. Those of the bacilli were investigated by SMITH *cs.* (103) and KNIGHT and PROOM (64). Recently VALERA and ALEXANDER (113) examined the requirements of 18 strains of denitrifying bacteria. Thus, many data have come available concerning the minimal requirements of most of the bacteria with a dissimilatory nitrate-reducing enzyme system. However, the conditions are scarcely established under which these bacteria possess an optimal activity of nitratase (the sequence of enzymatical reactions effecting the reduction of nitrate to gaseous products). In the experiments described in this chapter an attempt was made to trace these optimal conditions.

B. EXPERIMENTAL

1. *Media*

a. Nutritional requirements under aerobic conditions. For ascertaining the nutritional requirements of the denitrifying bacteria under aerobic conditions a basal medium was used, supplemented with different nitrogenous compounds and vitamins. The basal medium contained: KH_2PO_4, 900 mg; Na_2HPO_4, 1190 mg; $MgSO_4.7\ H_2O$, 500 mg; $CaCl_2.6\ H_2O$, 280 mg; Fe, 10 mg as Fe-sequesterene; $MnSO_4.\ 5\ H_2O$, 3 mg; $ZnSO_4.7\ H_2O$, 0.25 mg; $CuSO_4.5$

H_2O, 0.25 mg; H_3BO_3, 0.25 mg; $Na_2MoO_4.2\ H_2O$, 0.05 mg; distilled water, 1 litre. From this basal medium the following media were derived:

Medium b. Basal medium supplemented with 1 g KNO_3 per litre.
Medium c. Basal medium supplemented with 1 g $(NH_4)_2SO_4$ per litre.
Medium a. Basal medium supplemented with vitamin-free casamino acids, 1 g; tryptophane, 50 mg; cystine, 10 mg per litre.
Medium cv. Basal medium supplemented with $(NH_4)_2SO_4$, 1 g and 7 B-vitamins: biotin, 1 µg; folic acid, 2 µg; riboflavin, 100 µg; thiamine, 500 µg; nicotinic acid, 500 µg; pyridoxine-HCl, 500 µg; Ca-pantothenate, 500 µg per litre.
Medium av. Basal medium supplemented with amino acids and B-vitamins in amounts equal to those of media a and cv.

Test tubes, 18 mm in diameter were filled with 5 ml of the media and autoclaved. To these tubes 0.1 ml of a separately sterilized 5 per cent glucose solution was added, making the final glucose concentration 1000 mg per litre. The tubes were then inoculated with one drop of a very dilute suspension of the denitrifying bacteria and incubated at 28 °C, being placed in a slanting position to permit sufficient aeration. The growth was estimated qualitatively after different periods of time.

b. Nutritional requirements under anaerobic conditions. For studying the nutritional requirements under anaerobic conditions media were used different from those described above.

Medium B. Basal medium, identical to medium b.
Medium C. KH_2PO_4, 1.5 g; $(NH_4)_2HPO_4$, 7 g; $MgSO_4.7\ H_2O$, 0.5 g; distilled water 1 litre. Minor elements were added in quantities identical to those of the basal medium.
Medium A. K_2HPO_4, 5 g; NaCl, 1 g; vitamin-free casamino acids, 1.25 g; tryptophane, 100 mg; cystine, 15 mg; distilled water 1 litre.
Medium CV. Medium C supplemented with B-vitamins in amounts equal to those of medium cv.
Medium AV. Medium A supplemented with B-vitamins in amounts equal to those of medium cv.

Test tubes, 16 mm in diameter were filled with 10 ml of the media and autoclaved. If the tubes were not used immediately after preparation, they were steamed before use to drive off the free oxygen and rapidly cooled. With each of the media half of the number of tubes was supplemented with 0.5 ml of a 20 per cent potassium-nitrate solution; all tubes received 0.2 ml of a 50 per cent glucose solution, making the final concentration of both compounds 1 per cent. Finally the tubes were inoculated and capped with 15 mm of melted vaspar. The bacteria were incubated at approximately the optimum temperature which was estimated separately. Growth was estimated qualitatively after different periods of time.

c. Effect of the carbon source. For studying the influence of various carbon sources on the denitrification process, a medium was used, described by SMITH *cs.* (103). The medium contained: tryptose, 10 g; beef extract, 3 g; yeast extract, 2 g; K_2HPO_4, 5 g; tap water, 1 litre. Approximately 10 ml of the medium were transferred to 16 mm tubes and autoclaved. If the tubes were not used immediately after preparation, they were steamed, cooled, and supplemented with aliquots of separately sterilized glucose, glycerol and Ca-lactate solutions to make the final concentration 1 per cent. Half of the tubes received 0.5 ml of a 20 per cent potassium-nitrate solution. Finally the tubes were inoculated, capped with 15 mm of sterile, melted vaspar, and incubated at the optimum temperature of the individual bacteria.

2. *Microorganisms*

The following species were tested in the media described above:

1. *Pseudomonas* str. 241, *Achromobacter* str. 181 and *B. macerans*, isolated from grassland sods incubated anaerobically with nitrate.

2. *B. cereus*, *B. cereus* var. *mycoides* *B. circulans*, *B. macerans*, *B. laterosporus*, *B. coagulans* and unidentified coccoid forms, which were all isolated from grassland sods under normal field conditions.

3. *Ps. aeruginosa*, *Sp. itersonii*, *Spirillum* str. 425 and *B. licheniformis*, isolated from enrichment cultures inoculated with grassland soil.

4. *Ps. stutzeri*, *Ps. denitrificans*, *M. denitrificans and B. polymyxa*, obtained from the culture collection of the Laboratory of Microbiology, Wageningen.

C. Results

1. *Nutritional requirements*

The nutritional requirements of the denitrifying bacteria isolated during the experiments described in the preceding chapter, were estimated aerobically, and anaerobically with and without nitrate (Table 27). The total collection clearly broke up in two distinct groups. The organisms of the first group, of which *Ps. aeruginosa* was a representative, were able to grow under anaerobic conditions only when nitrate was present in the medium. The organisms of the second group, of which *B. licheniformis* was a representative, developed under anaerobic conditions also in the absence of nitrate. Except in the case of *B. polymyxa* and *B. macerans* (which are known to produce carbon dioxide from carbohydrates under anaerobic conditions), formation of gas by representatives of the second group was observed only in the media containing nitrate. Although it is not certain that the gas produced under these conditions consisted entirely of reduction products of nitrate, it is clear that the metabolic pattern was modified by nitrate.

The second difference between the bacteria of the *Ps. aeruginosa*-group and those of the *B. licheniformis*-group, is that the dissimilatory reduction of nitrate by the former proceeded very rapidly and usually was completed within a few days. The second group, except *Spirillum* str. 425 and *Achromobacter* str. 181, reduced nitrate slowly, nitrate sometimes being still present in the medium after 14 days' incubation.

In separate experiments it was found that the organisms of the *Ps. aeruginosa*-group transformed 20 to 25 per cent of the glucose into cell material under anaerobic conditions in the presence of nitrate, while under the same conditions the dry-matter production by the species of the *B. licheniformis*-group seldom exceeded 6 per cent of the weight of the added glucose.

Although considerable differences obviously existed between the various organisms tested, attention was mainly focussed on their ability to transform nitrate to gaseous products. The influence of amino acids on this process was especially considered. From the data presented in Table 27 it appears that, generally, the nutritional requirements of the various bacteria were the same under both aerobic and anaerobic conditions. *Spirillum* str. 425 and *B. licheniformis*, however, required amino acids for growth under anaerobic but not under aerobic conditions. The results obtained with *B. licheniformis* are in accordance with the findings of Verhoeven (115) and Valera and Alexander (113). *Sp. itersonii*, *Achromobacter* str. 181 and *B. cereus* were dependent on amino acids, under anaerobic as well as under aerobic conditions. This was also true for *Ps. stutzeri*, an observation which was already recorded by Valera and Alexander (113). This organism, however, was found capable of growing in a mineral medium with ammonium sulphate as a nitrogen source, when alcohols or organic acids were used as a source of carbon and energy instead of carbohydrates. The other *Pseudomonas*-species also developed in the mineral medium supplemented with glucose, as did *M. denitrificans*, both aerobically and anaerobically in the presence of nitrate. The statement by Valera and Alexander (113), that the latter organism also requires amino acids, was not confirmed.

B. circulans, *B. laterosporus*, *B. macerans*, and *B. polymyxa* required B-vitamins for growth under both aerobic and anaerobic conditions and with in-

TABLE 27. The nutritional requirements of different species of denitrifying bacteria.

Species	Number of strains	Opt. temp.	Aerobic					Anaerobic					Anaerobic + nitrate				
			b	c	a	cv	av	B	C	A	CV	AV	B	C	A	CV	AV
Ps. aeruginosa	1	28 °C	+	+	+	+	+	—	—	—	—	—	+G	+G	+G	+G	+G
Ps. stutzeri	1	28 °C	—	—	+	—	+	—	—	—	—	—	—	—	+G	—	+G
Ps. denitrificans	1	28 °C	+	+	+	+	+	—	—	—	—	—	+G	+G	+G	+G	+G
Pseudomonas str. 241	2	28 °C	+	+	+	+	+	—	—	—	—	—	+G	+G	+G	+G	+G
M. denitrificans	1	28 °C	+	+	+	+	+	—	—	—	—	—	+G	+G	+G	+G	+G
Sp. itersonii	1	28°C	—	—	+	—	+	—	—	—	—	—	—	—	+G	—	+G
Achromobacter str. 181	2	28 °C	—	—	+	—	+	—	—	+	—	+	—	—	+G	—	+G
Spirillum str. 425	1	28 °C	—	—	+	+	+	—	—	+	—	+	—	—	+G	—	+G
B. licheniformis str. Pn[1]	1	37 °C	+	+	+	+	+	—	—	+	—	+	—	—	+	—	+
B. licheniformis str. 434	1	37 °C	+	+	+	+	+	—	—	+	—	+	—	—	+G	—	+G
B. licheniformis	3	37 °C	+	+	+	+	+	—	—	+	—	+	—	—	+(G)	—	+(G)
B. circulans	3	40 °C	—	—	—	+	+	—	—	—	+	+	—	—	—	+G	+G
B. laterosporus	1	37 °C	—	—	—	+	+	—	—	—	+	+	—	—	—	+G	+G
B. coagulans	1	37 °C	—	—	—	—	+	—	—	—	—	+	—	—	—	—	+G
B. cereus	20	28 °C	—	—	+	—	+	—	—	+	—	+	—	—	+	—	+
B. cereus var. mycoides	8	28 °C	—	—	+	—	+	—	—	+	—	+	—	—	+	—	+
B. macerans	3	37 °C	—	—	—	+	+	—	—	—	+G	+G	—	—	—	+G	+G
B. polymyxa	1	37 °C	—	—	—	+	+	—	—	—	+G	+G	—	—	—	+G	+G
Coccoid forms	5	28 °C	—	—	—	—	+	—	—	—	—	+	—	—	—	—	+

[1] Isolated and kindly supplied by Dr W. Verhoeven, who has used this strain in his investigations (115).

— no growth
\+ positive growth
G gas production

b basal medium with nitrate
B basal medium without nitrate
c, C mineral media with ammonium nitrogen
a, A mineral media with amino acids
cv, CV mineral media with ammonium nitrogen and B-vitamins
av, AV mineral media with amino acids and B-vitamins

organic- or organic-nitrogen sources. The same nutritional requirements of these species under aerobic conditions were found by KNIGHT and PROOM (64). *B. coagulans* and the coccoid forms required both amino acids and B. vitamins. The latter forms, *B. cereus* and some *B. licheniformis*-strains did not show gas production from nitrate in the media adopted for the tests. However, in more complex media containing tryptose, beef extract and yeast extract, the latter two species were able to form gaseous products from nitrate (Table 29).

2. *Influence of amino acids on denitrification rate*

Although amino acids appeared to be required by some of the denitrifying bacteria, these compounds did not seem to be of primary importance for others. Moreover, the species dependent on amino acids, except *Achromobacter* str. 181, were not involved in the denitrification process in the grass sods. *Pseudomonas* str. 241 which was the main representative of the denitrifying population of the grass sods showed growth and vigorous gas production in the basal medium supplemented with glucose and nitrate.

A different picture of the influence of amino acids on denitrification was obtained, when attention was paid to the rate of gas production by the bacteria not requiring amino acids for growth (Table 28). Both growth rate and rate of gas production of the organisms of the *Ps. aeruginosa*-group were strongly stimulated by the presence of amino acids in the medium. On the other hand, only the growth rate of *B. macerans, B. polymyxa, B. circulans* and *B. laterosporus* (*B. licheniformis*-group) was increased by amino acids but not the rate of gas production. This might indicate that amino acids had a favourable effect on the rate of production of cell material of both groups of denitrifying bacteria, and in addition, stimulated the reduction rate of nitrate to gaseous products by the species of the *Ps. aeruginosa*-group. The latter could be explained by assuming that amino acids constitute a more favourable type of hydrogen donor for these organisms than carbohydrates. This hypothesis will be discussed in more detail in the next chapter.

The results of the above experiment demonstrate that amino acids increase the rate of growth and gas production of many of the tested denitrifying bacteria. Some of these organisms required the compounds as indispensable nutrients for growth, while others were merely stimulated. Therefore, it is likely that the nutritional conditions are more optimal for denitrification in the root environment of plants, which excrete amino acids, than in the rest of the soil. The high denitrification rate observed when compounds supplied by pea roots were used, may be due to their amino-acid excretion.

Although the effect of amino acids on denitrification was obvious, it was still not understood why some species, like *Pseudomonas* str. 241, were preferentially stimulated during the denitrification process in the soil, whereas others, like *B. cereus* and *B. licheniformis,* were not affected. As already mentioned, the production of cell material of the latter species, under anaerobic conditions in the presence of nitrate, was very low compared with that of the species of the *Ps. aeruginosa*-group. This may have resulted in a low competition capacity of the bacilli, when both groups were present in the same environment. The next chapters will show that the capacity of the bacilli to compete with other denitrifying organisms is weak for other reasons as well.

TABLE 28. The influence of amino acids on growth and gas production of denitrifying bacteria under anaerobic conditions in the presence of nitrate.

Species	Medium	Time (days)				
		2	3	5	7	17
Ps. aeruginosa	B	—	—	—	+G	+G
	C	—	+G	+G	+G	+G
	CV	+	+G	+G	+G	+G
	A	+G	+G	+G	+G	+G
	AV	+G	+G	+G	+G	+G
Ps. denitrificans	B	—	—	—	—	+G
	C	—	—	+G	+G	+G
	CV	—	+G	+G	+G	+G
	A	+G	+G	+G	+G	+G
	AV	+G	+G	+G	+G	+G
Pseudomonas str. 241	B	—	—	—	—	+G
	C	—	—	—	+G	+G
	CV	—	+	+G	+G	+G
	A	+G	+G	+G	+G	+G
	AV	+G	+G	+G	+G	+G
M. denitrificans	B	—	—	—	—	+G
	C	—	—	+	+G	+G
	CV	+	+	+G	+G	+G
	A	+G	+G	+G	+G	+G
	AV	+G	+G	+G	+G	+G
B. macerans	CV	—	—	+	+G	+G
	AV	+	+	+	+G	+G
B. polymyxa	CV	—	—	—	+G	+G
	AV	+	+	+	+G	+G
B. circulans	CV	—	—	+	+G	+G
	AV	+	+	+	+G	+G
B. laterosporus	CV	—	—	+	+	+G
	AV	+	+	+	+	+G

\+ positive growth
— no growth
G gas production

B Basal medium
C Mineral medium with ammonium nitrogen
CV Mineral medium with ammonium nitrogen and B-vitamins
A Mineral medium with amino acids
AV Mineral medium with amino acids and B-vitamins

3. *Effect of the carbon source on growth of organisms of the B. licheniformis-group*

When the results were discussed of the experiments in which representatives of the *B. licheniformis*-group were tested for their denitrifying capacity, these organisms were considered to perform a dissimilatory nitrate reduction (see preceding chapter), since growth occurred under anaerobic conditions only with nitrate added. In that test glycerol was used as a carbon source. In the experiment described above (Table 27), however, the presence of nitrate was not required to enable growth of these bacteria under anaerobic conditions; in that experiment glucose was the carbon source. To clear up this discrepancy,

the effect of different carbon sources on the growth of the bacteria of the *B. licheniformis*-group was examined under anaerobic conditions. As it was shown by VERHOEVEN (115) that *B. licheniformis* denitrified only in the presence of glucose, glycerol, or lactate, these compounds were selected for the experiment (Table 29). It is obvious that only using glucose as a carbon source, nitrate was not required for growth under anaerobic conditions; its presence may even cause lower yields of cell material. With glycerol and lactate, however, generally no growth occurred under such conditions. Some species gave a slight growth with glycerol in the absence of nitrate.

TABLE 29. Effect of the carbon source on the growth of the organisms of the *B. licheniformis*-group under anaerobic conditions in a complex medium containing tryptose, beef extract and yeast extract.

Species	Time (days)	Glucose		Glycerol		Lactate		No carbon source	
		–	NO_3^-	–	NO_3^-	–	NO_3^-	–	NO_3^-
B. licheniformis str. 434	3	8	3G	0	3G	0	3G	0	1
	10	8	2G	0	1G	0	+G	0	+G
B. licheniformis str. P 1[1]	3	4	1	0	2	0	1	0	1
	10	4	2G	0	1G	0	1G	0	1G
B. circulans str. 439 B	3	4	4	0	2	0	+	0	2
	10	4	2G	0	3G	0	+	0	2
B. circulans str. 465 x	3	4	4G	+	3G	0	+	0	3
	10	4	2G	2	3G	0	+	0	2
B. laterosporus str. 468 B	3	4	3G	2	2	0	1	0	+
	10	3	3G	2	3G	0	1	0	1
B. coagulans str. 465 A	3	6	4	+	2	0	1	0	1
	10	5	3G	+	2G	0	1	0	1
B. cereus str. 372	3	3	4	+	3	+	3	+	2
	10	3	4G	+	3G	+	3G	+	2G
B. macerans str. 408	3	6G	3G	1	3	0	+	0	+
	10	6G	2G	1	2G	0	+G	0	+
B. polymyxa str. 3	3	5G	3	2G	3	0	1	0	+
	10	3G	2G	2G	3G	0	1	0	1
Spirillum str. 425	3	4	4G	1	4G	0	4G	0	4G
	10	6	6G	1	4G	0	5G	0	2G
Achromobacter str. 181	3	6	4G	0	3G	0	2	0	2
	10	6	4G	0	3G	0	2G	0	2G
Coccoid forms str. 471	3	2	2	0	2	0	2	0	2
	10	2	2	0	2	0	2	0	2

[1] Isolated and kindly supplied by Dr. W. Verhoeven, who has used this strain in his investigations (115).

0 no growth
+ doubtful growth
1–8 rising amounts of growth
G gas production

D. Discussion

VERHOEVEN (115) distinguished two different types of dissimilatory nitrate reduction: a. incidental dissimilatory nitrate reduction in which the nitrate merely acts as a non-essential hydrogen acceptor, b. true dissimilatory nitrate reduction in which the nitrate acts as a hydrogen acceptor essential for growth. *B. licheniformis,* tested by VERHOEVEN only in a medium with glycerol as the carbon source, was assumed to possess the latter type of nitrate reduction. The results of the present investigation (Table 29) show that according to their growth on glycerol and lactate, the organisms of the *B. licheniformis*-group indeed should be reckoned among the true denitrifying bacteria. According to their growth on glucose, however, these organisms should be regarded as incidental denitrifyers.

It is a well-known fact that the tested *Bacillus*-species may ferment glucose; e.g. *B. licheniformis* produces lactate, glycerol and acetylmethylcarbinol as the main products from glucose (11). The restricted growth of most of the organisms with glycerol and especially with lactate under anaerobic conditions in the presence of nitrate, favours the hypothesis that nitrate under these conditions is only needed to enable fermentation of these compounds by achieving their first oxidation steps. Therefore, the importance of nitrate is limited and cannot be compared with its function in *Ps. aeruginosa,* where it brings about the complete degradation of glucose to carbon dioxide and water (116).

In those cases, where, with glycerol as the carbon source, growth occurred also in the absence of nitrate, the large quantiy of amino acids present in the medium, may have taken over the function of nitrate as a hydrogen acceptor in a way, similar to that observed with some organisms of the *E. coli*-group (12). At the end of the experimental part of the present investigation, an *E. coli*-strain was isolated which also needed nitrate for growth under anaerobic conditions in a peptone-containing medium with glycerol as the carbon source. This strain was not included in the investigation.

The results of Table 29 explain the observation of VALERA and ALEXANDER (113), who counted the highest numbers of denitrifying organisms in the soil when glycerol was used as a carbon source in the test medium. Considerably lower numbers were found using glucose.

Although it appeared that nitrate was required only under certain conditions by the organisms of the *B. licheniformis*-group, no information was obtained concerning the activity of the nitrate-reducing enzymes under these conditions. This subject will be discussed in chapters IX and X where an attempt will be made to discover why the organisms of the *Ps. aeruginosa*-group are preferentially stimulated during soil denitrification in competition with those of the *B. licheniformis*-group.

E. Summary

By investigating the nutritrional requirements of the isolated denitrifying bacteria, it was shown that amino acids are indispensable for growth of some of these organisms and exert a stimulative effect on both growth and gas production of others. It appeared that the denitrifying organisms can be divided into two groups. The *Ps. aeruginosa*-group always required nitrate under anaerobic conditions. The *B. licheniformis*-group was able to grow in the absence of nitrate when glucose was used as a carbon source, but needed this compound

when glucose was replaced by glycerol or lactate. It was suggested that in the latter case, nitrate was required to enable the oxydation of these compounds to dihydroxy acetone and pyruvic acid. Also in other respects large differences existed between both groups of organisms (slow denitrification and low production of cell material by the bacteria of the *B. licheniformis*-group as compared with those of the *Ps. aeruginosa*-group).

The low yields of cell material of most organisms of the *B. licheniformis*-group under anaerobic conditions in the presence of nitrate may indicate that, during denitrification in soil, this group is at a disadvantage in the competition with organisms of the *Ps. aeruginosa*-group.

CHAPTER IX

THE EFFECT OF DIFFERENT HYDROGEN DONORS ON THE DISSIMILATORY NITRATE REDUCTION BY *PS. AERUGINOSA*

A. Introduction

In chapter VII *Pseudomonas*-species were shown to be involved in the denitrification process in permanent-grassland soils. The isolated species may use root excretions as the sole source of hydrogen donors during denitrification. It was supposed that particularly excreted amino acids constitute a favourable substrate for denitrifying organisms. The examination of the nutritional requirements of the *Ps. aeruginosa*-group demonstrated that amino acids were essential for the growth of some organisms and stimulated both growth rate and rate of gas production of the other species which were able to grow in a medium containing glucose, with nitrate as the sole nitrogen source.

In order to investigate in more detail the effect of amino acids on denitrification by the *Pseudomonas*-group, the influence of various hydrogen donors on denitrification by washed cells of *Ps. aeruginosa* was examined. The experiments pertaining to this subject are recorded below.

B. Literature

Few data are recorded in the literature concerning the effect of hydrogen donors on the denitrification rate of the organisms of the *Ps. aeruginosa*-group. Verhoeven and Goos (116), using glucose as a hydrogen donor, observed a complete dissimilation into carbon dioxide and water during denitrification by *Ps. aeruginosa*. Approximately twenty-five per cent of the added glucose were incorporated into cell material. Glutamate and succinate were also completely dehydrogenated by *Ps. denitrificans* during dissimilatory nitrate reduction (97), but only eight per cent of the added carbon source was converted into dry matter. According to Chang and Morris (23), cell yield of *M. denitrificans* under anaerobic conditions in the presence of nitrate was scarcely lower than that obtained under aerobic conditions. The results suggest that during denitrification by the organisms of the *Pseudomonas*-group the hydrogen donors are dehydrogenated in the same way as when oxygen is used as the terminal hydrogen acceptor.

The effect of various substrates on growth of *Ps. indigofera* was studied by

Mc FADDEN and HOWES (29). It appeared that growth under anaerobic conditions in the presence of nitrate was more rapid with aspartic acid than with succinate.

C. EXPERIMENTAL

To investigate the effect of different hydrogen donors on the denitrification rate of washed cells of *Ps. aeruginosa*, the organism was cultivated in 300 ml Erlenmeyer flasks containing 100 ml of the following media:

1. Tryptose, 10 g, beef extract, 3 g; yeast extract, 2 g; glucose, 10 g; K_2HPO_4, 5 g; KNO_3, 10 g; tap water, 1 litre.
2. Identical to medium 1, but glucose omitted.
3. Glucose, 10 g; KNO_3, 10 g; K_2HPO_4, 1 g; $MgSO_4.7\ H_2O$, 0.5 g; $CaCl_2.6\ H_2O$, 20 mg; Fe as Fe-sequesterene, 10 mg; $MnSO_4.5\ H_2O$, 3 mg; $ZnSO_4.7\ H_2O$, 0.25 mg; $CuSO_4.5\ H_2O$, 0.25 mg; H_3BO_3, 0.25 mg; $Na_2MoO_4.2\ H_2O$, 0,05 mg; distilled water, 1 litre.

The flasks were inoculated with a culture maintained on peptone-glycerol-nitrate-agar slopes, and incubated at 28 °C in a nitrogen atmosphere. The cells were harvested in the exponential phase, washed once with distilled water and once with 0.05 M phosphate-salts buffer, pH 7.0. Subsequently the cells were re-suspended in 1/15 M phosphate-salts buffer pH 7.0. Two ml of the cell suspensions, which contained approximately 3 mg dry matter per ml solution, were transferred to Warburg vessels containing 0.3 ml of a 10 per cent KOH-solution in the center well. The air inside the vessels was replaced by oxygen-free gaseous nitrogen. Varying amounts of glucose, α-ketoglutaric acid and glutamic acid were added separately or in combination as hydrogen donors. Nitrate and nitrite were used as hydrogen acceptors. Gas production was estimated manometrically at 28 °C. Although the gas may have consisted of a small quantity of nitrous oxide, apart from gaseous nitrogen (116), the greater solubility of the former gas was neglected. The gas production was expressed as μ mole gaseous nitrogen. Nitrate, nitrite and ammonium-nitrogen content of the solutions were estimated periodically by analysing duplicate vessels.

Since the uptake of α-ketoglutaric acid by the cells proceeded less rapidly than that of glutamic acid, some additional experiments were performed with frozen cells in order to eliminate the selective permeability of the cell membrane. Dense suspensions of washed cells were frozen for 5 minutes in a solid carbon dioxide-alcohol mixture and then rapidly thawed. This procedure was repeated four times. Experiments were carried out with these cells, identical to those executed with normal washed cells.

During the experiments, organic acids were estimated qualitatively in the medium. For that purpose the cells were centrifugated. The acidified centrifugate was extracted with ether for 48 hours. After distillation of the ether, the residue was diluted by adding a small quantity of water, and acidified. The non-volatile organic acids were estimated by two-dimensional paper chromatography, using a method described by HEITFUSS and WOLFGANG (50).

The formation of intermediary nitrite in growing cultures with various carbon sources was studied in a mineral medium which had the following composition: K_2HPO_4, 5 g; KNO_3, 1 g; $MgSO_4.7\ H_2O$, 0.2 g; minor elements in the same quantities as in Medium 3 described above; carbon source, 4 g; distilled water, 1 litre. Tubes were filled with 10 ml of the medium, autoclaved, inoculated and incubated at 28 °C in a nitrogen atmosphere.

D. RESULTS

1. *Comparison of glucose and glutamic acid as hydrogen donors*

The effect of glucose, glutamic acid, separate and in combination on the dissimilatory nitrate reduction of washed cells of *Ps. aeruginosa*, grown under anaerobic conditions in the presence of nitrate, is shown in Fig. 6. Since the washed cells had been adapted to nitrate dissimilation, the gas production started without a lag period. Gaseous nitrogen was produced at the same rate as nitrate was consumed, indicating that no intermediary compounds accumulated. The gas production ceased immediately after the nitrate was exhausted. Without added hydrogen donors, no denitrification occurred. Nitrite and ammonia were not detected at any stage of the experiment. The latter observation was in accordance with the results obtained by VERHOEVEN and

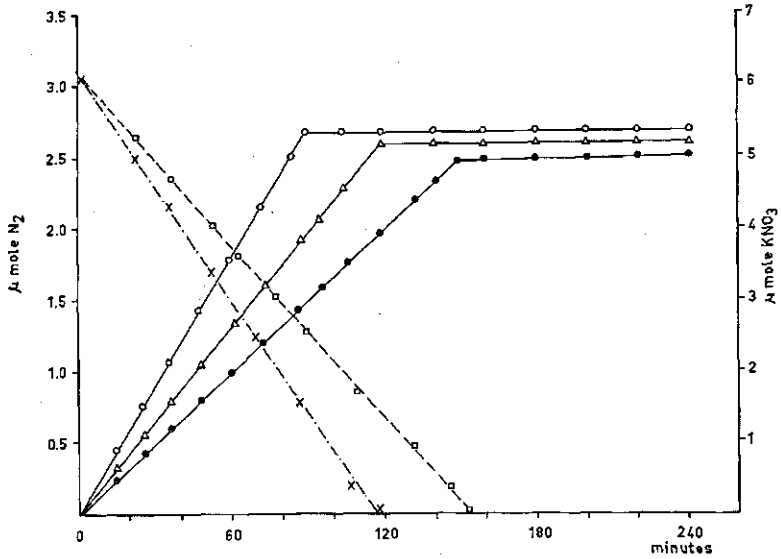


FIG. 6. Production of gas and consumption of nitrate during denitrification by washed cells of *Ps. aeruginosa*, grown in Medium 1. The cell suspension was supplied with different hydrogen donors. A. Production of gas, expressed as N_2, μ mole per ml. ●—● 2.45 μ mole glucose + 6 μ mole KNO_3 per ml; △—△ 4.75 μ mole glutamic acid + 6 μ mole KNO_3 per ml; o—o 2.45 μ mole glucose + 4.75 μ mole glutamic acid + 6 μ mole KNO_3 per ml. B. consumption of nitrate, μ mole per ml. □- - - -□ glucose; ×-·-·-× glutamic acid.

GOOS (116). SACHS and BARKER (96), using acetate as a hydrogen donor in denitrification, observed a temporary accumulation of nitrite by washed cells of *Ps. denitrificans*. Washed cells of *Ps. aeruginosa*, grown in the medium used by SACHS and BARKER, produced only a neglegible quantity of intermediary nitrite using acetate as a hydrogen donor.

The rate of gas production using glutamic acid as a hydrogen donor was considerably higher than that using glucose. The combination of both hydrogen donors increased the rate even more. Since in additional experiments the concentrations of the separate hydrogen donors in the range used in this experiment appeared to have no influence on denitrification rate (Fig. 7), it was concluded that glutamic acid is superior to glucose as a hydrogen donor. Similar results were obtained with other amino acids (alanine, lysine, vitamin-free casamino acids) which always effected a higher denitrification rate than carbohydrates.

Because the combination of glucose and glutamic acid brought about a higher denitrification rate than the separate compounds (Fig. 6), the nitratase activity obviously was not limiting the rate of gas production in the case of the separate compounds. The lower denitrification rates with the separate compounds may indicate that either the rate of uptake by the cells, or the activity of the enzymes involved in the dehydrogenation of glucose and glumatic acid, respectively, is limiting the process. Since cells in which the selective uptake was eliminated by alternately freezing and thawing, gave results, identical to those presented in Table 6, the former hypothesis is unlikely.

To demonstrate that dehydrogenation of glutamic acid and glucose are

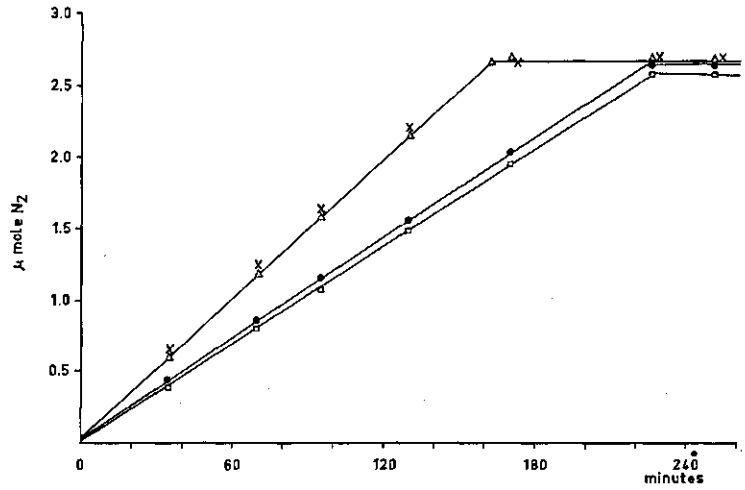


FIG. 7. Production of gas (expressed as N_2, μ mole per ml) during denitrification by washed cells of *Ps. aeruginosa*, grown in Medium 1; the cell suspension was supplied with different quantities of glucose and glutamic acid. ●—● 2.45 μ mole glucose + 6 μ mole KNO_3 per ml; □—□ 5.50 μ mole glucose + 6 μ mole KNO_3 per ml; △—△ 4.75 μ mole glutamic acid + 6 μ mole KNO_3 per ml; ×—× 9.50 μ mole glutamic acid + 6 μ mole KNO_3 per ml.

limiting the denitrification rate, some experiments were performed with nitrite as a hydrogen acceptor. As compared with nitrate less hydrogen will be required to reduce this compound to nitrogen gas:

$$2\ HNO_3 + 10\ H \longrightarrow N_2 + 6\ H_2O$$
$$2\ HNO_2 + 6\ H \longrightarrow N_2 + 4\ H_2O$$

Therefore, if the dehydrogenase activity is limiting the denitrification rate, a higher rate of gas production may be expected when nitrate is used as a hydrogen acceptor than in the case of nitrite. The experiments in which nitrite and nitrate were compared as a hydrogen acceptor are recorded below.

2. *Comparison of nitrite and nitrate as a hydrogen acceptor*

It appeared that with both glucose and glutamic acid the denitrification rate using nitrite was considerably higher than that using nitrate (Fig. 8). Therefore it was concluded that under the experimental conditions the dehydrogenase activity was limiting the rate of gas production from nitrate. Similar results were obtained by ALLEN and VAN NIEL (1) and explained similarly.

In most experiments, over 85 per cent of the added nitrate was reduced to nitrogen gas, whereas in the case of nitrite this percentage varied between fifty and seventy per cent. No attempts were made to trace the fate of the nitrite nitrogen not recovered as gaseous nitrogenous products.

Curiously, the effects of glucose and glutamic acid on the reduction rate of nitrite were the reverse of those observed with nitrate. In the presence of nitrite the denitrification rate was considerably higher with glucose than with glutamic acid. This led to the conclusion that glutamic acid as compared with glucose was a superior hydrogen donor only in the reduction from nitrate to nitrite. Since the latter step limits the denitrification rate (nitrite did not accu-

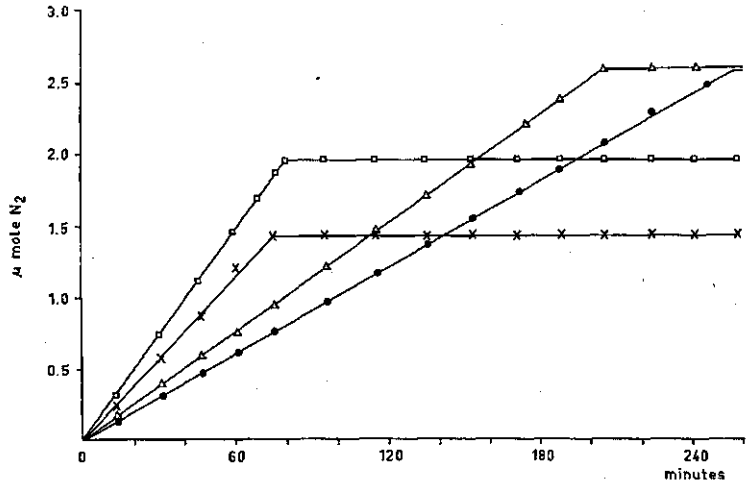


FIG. 8. Production of gas (expressed as N_2, μ mole per ml) during denitrification by washed cells of *Ps. aeruginosa*, grown in Medium 1; the cell suspension was supplied with glucose and glutamic acid as hydrogen donors and with nitrate and nitrite as hydrogen acceptors. ●—● 2.45 μ mole glucose + 6 μ mole KNO_3 per ml; □—□ 2.45 μ mole glucose + 6 μ mole KNO_3 per ml; △—△ 4.75 μ mole glutamic acid + 6 μ mole KNO_3 per ml; ×—× 4.75 μ mole glutamic acid + 6 μ mole KNO_3 per ml.

mulate), the whole denitrification process proceeds more rapidly with glutamic acid than with glucose. The fact that glutamic acid was superior to glucose in the reduction of nitrate to nitrite, whereas the reverse was observed in nitrite reduction, may be attributed to the existence of different hydrogen-transporting systems in nitrate and nitrite reduction. Investigations with *Ps. aeruginosa,* which showed that DPNH was involved in the reduction of nitrate to nitrite (33) but could not be used as an electron donor in the subsequent reduction of nitrite (124), may support this hypothesis.

3. *Comparison of glutamic acid and α-ketoglutaric acid as hydrogen donors using nitrate as a hydrogen acceptor*

Since α-ketoglutaric acid is an intermediate in the breakdown of both glucose and glutamic acid, some experiments were performed with this compound as a hydrogen donor. It appeared, however, that, with washed cells, the rate of gas production from nitrate and the rate of oxygen uptake in an aerobic experiment, were about 40 per cent of those when glucose was used. This result presumably was due to a slow uptake of α-ketoglutaric acid by the intact cells. To eliminate the selective uptake by the cells, the experiment was repeated with alternately frozen and thawed cells (Fig. 9). The denitrification rate using glutamic acid proved to be higher than that using α-ketoglutaric acid. Under aerobic conditions, however, oxygen was consumed at nearly the same rate with each of both compounds. Using glutamic acid as a hydrogen donor during denitrification, α-ketoglutaric acid was accumulated in the medium.

On repeating the experiment with a crude cell extract, obtained by using a MSE sonic oscillator, no gaseous reduction products were observed; in the presence of glumatic acid 1 μ mole nitrite was produced, accompagnied by

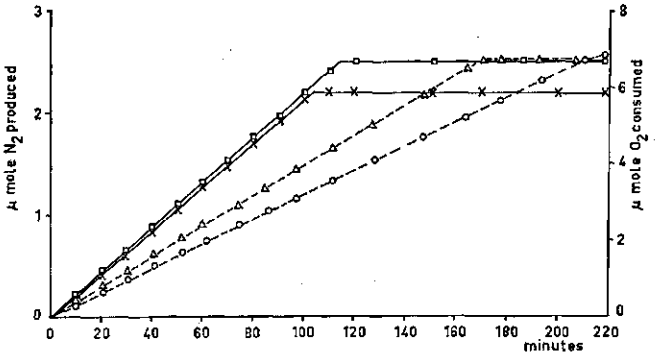


FIG. 9. Oxygen uptake and production of gas (expressed as N_2, μ mole per ml) during the dehydrogenation of glutamic acid and α-ketoglutaric acid by alternately frozen and thawed cells of *Ps. aeruginosa* (8 mg dry matter per ml), grown in Medium 1; oxygen and nitrate were used as terminal hydrogen acceptors □——□ oxygen uptake with 4.75 μ mole glutamic acid per ml; ×——× oxygen uptake with 4.25 μ mole α-ketogluteric acië per ml; △ - - - - △ nitrogen production with 4,75 μ mole glutamic acid + 6 μ mole KNO_3 per ml; o - - - - o nitrogen production with 4.25 μ mole α-ketoglutaric acid + 6 μ mole KNO_3 per ml.

an accumulation of α-ketoglutaric acid and some succinic acid. Using α-ketoglutaric acid as the substrate, 0.2 μ mole nitrite was formed along with a small amount of succinic acid. No organic acids were produced from glutamic acid or α-ketoglutaric acid, when nitrate was omitted. These results, which were repeatedly obtained, support the hypothesis that the favourable effect of glutamic acid as a hydrogen donor during denitrification may be attributed to the activity of glutamic-acid dehydrogenase which effects a more rapid hydrogen transport to nitrate than the dehydrogenase systems involved in the dissimilation of glucose and α-ketoglutaric acid.

4. *Effect of differences of the precultivation medium on the denitrification rate of washed cells*

When glucose was used as a hydrogen donor in an experiment with washed cells which had been grown in a medium without glucose (Medium 2), an extended lag was observed before the production of gaseous nitrogen started (Fig. 10). Apparently the enzymes involved in the hydrogen transport from glucose to nitrate, were not present as constitutive enzymes. When, however, the washed cells had been grown in a medium containing no amino acids (Medium 3), glutamic acid could be used as a hydrogen donor without any lag period, indicating that the cells contained the responsible dehydrogenases as constitutive enzymes, although their activity had considerably decreased (Fig. 11). From these results it may be concluded that under natural conditions (e.g. in soil) *Ps. aeruginosa* may use glutamic acid more readily than glucose as a hydrogen donor in denitrification.

5. *Root systems as a source of hydrogen donors*

In an experiment with root systems of sterile pea plants, a heavy suspension of washed cells of *Ps. aeruginosa*, grown in Medium 1. was added to the roots. It appeared that denitrification did not start immediately; during the experiment a considerable accumulation of nitrite was observed. This was in con-

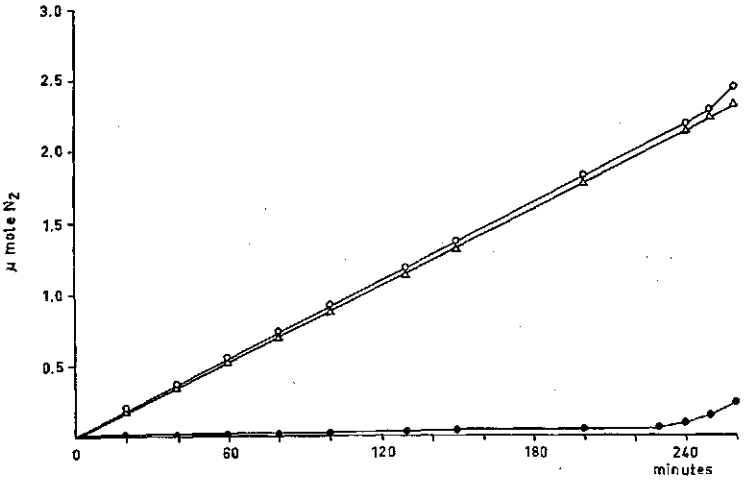


FIG. 10. Production of gas (expressed as N_2, μ mole per ml) during denitrification by washed cells of *Ps. aeruginosa*, grown without glucose (Medium 2); the cell suspension was supplied with different hydrogen donors. ●—● 2.45 μ mole glucose + 6 μ mole KNO_3 per ml; △ — △ 4.75 μ mole glutamic acid + 6 μ mole KNO_3 per ml; o—o 2.45 μ mole glucose + 4.75 μ mole glutamic acid + 6 μ mole KNO_3 per ml.

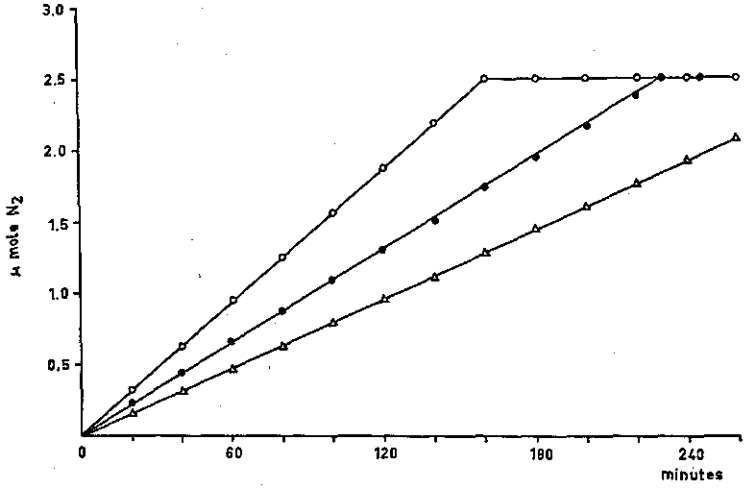


FIG. 11. Production of gas (expressed as N_2, μ mole per ml) during denitrification by washed cells of *Ps. aeruginosa*, grown without glutamic acid (Medium 3); the cell suspension was supplied with different hydrogen donors. ●—● 2.45 μ mole glucose + 6 μ mole KNO_3 per ml; △ — △ 4.75 μ mole glutamic acid + 6 μ mole KNO_3 per ml; o—o 2.45 μ mole glucose + 4.75 μ mole glutamic acid + 6 μ mole KNO_3 per ml.

trast with the experiments with washed cells described above, in which nitrite was never observed as an intermediate. It was supposed that the accumulation of nitrite in the experiment with root systems was due to the presence of oxygen inclusions between the vermiculite particles of the root-artificial soil medium, which could have altered the relative activities of the nitrate and nitrite reductases. To test the effect of oxygen on nitrite formation, an experiment

with washed cells was carried out in Warburg vessels with partial oxygen pressures ranging from 0.1 to 20 per cent. No nitrite accumulation was observed at any of these pressures, showing that the presence of air inclusions in the root-soil medium cannot have been responsible for the observed nitrite accumulation in this medium.

In experiments with washed cell suspensions of *Ps. aeruginosa,* nitrite formation was observed only after re-suspension, when the cells started to grow. Table 30 gives the results of an experiment in which cells, grown in Medium 1, were transferred to complete media with different carbon sources. In most cases nitrite was formed in the early stages of growth. From these results it was concluded that the accumulation of nitrite in the experiment with pea roots, was the result of growth of the bacteria.

TABLE 30. Nitrite formation by growing cultures of *Ps. aeruginosa* in a mineral medium with different carbon sources.

Carbon source	Days	Growth	NO_2-formation	Carbon source	Days	Growth	NO_2-formation
Glutamic acid	1	—	+	Acetate	1	—	—
	3	+	+		3	—	+
Asparagine	1	—	+	Pyruvate	1	—	—
	3	+	+		3	—	—
Aspartic acid	1	—	+	Lactate	1	—	—
	3	+	+		3	+	+
Casamino	1	+	disappeared	Maltose	1	—	—
acids	3	+	disappeared		3	+	—
Fumarate	1	—	+	Galactose	1	—	—
	3	+	+		3	+	+
Citrate	1	—	—	Glucose	1	—	—
	3	+	+		3	+	+
Succinate	1	—	—	Ethanol	1	—	—
	3	—	+		3	+	+
				Control	1	—	—
					3	—	—

E. DISCUSSION

The results, obtained with washed cells of *Ps. aeruginosa,* agree with the conception that amino acids promote denitrification in the soil. The stimulative effect may be brought about by relatively high activities of the amino-acid dehydrogenases during denitrification and the ability of the bacteria to use amino acids without an adaptation period. Results of BROADBENT (19) demonstrate that during denitrification in soil ammonification proceeded at the same rate as in air, whereas carbon-dioxide production had considerably decreased. These results can also be attributed to the fact that during denitrification desaminating activities are relatively high but that further breakdown is retarded.

The arguments in favour of the stimulative effect of amino acids on denitrification may be summarized as follows:

1. Denitrification is stimulated by root excretions of living plants.
2. Root excretions of pea plants effect a higher denitrification rate than those of perennial ryegrass.
3. Sterile pea plants excrete more amino acids than perennial ryegrass (excretion of amino acids by non-sterile peas cannot be estimated).

4. The bacteria involved in the denitrification process in the soil may use root excretions of pea plants as the sole source of hydrogen donors during denitrification.
5. Amino acids stimulate growth rate and rate of gas production from nitrate of the bacteria involved in the denitrification process in the soil.
6. Amino acids proved to be superior to carbohydrates as a source of hydrogen donors in experiments with washed cells of *Ps. aeruginosa,* a species which was chosen as the type species of the *Pseudomonas*-group. In contrast with glucose, the cells were able to use amino acids without an adaptation period.

F. Summary

A study was made of the effect of glucose, α-ketoglutaric acid and glutamic acid as hydrogen donors in denitrification by washed cells of *Ps. aeruginosa.* Glutamic acid appeared to be superior to the other compounds as a hydrogen donor. This was probably due to the high activity of glutamic-acid dehydrogenase in denitrification. An accumulation of α-ketoglutaric acid was observed during denitrification using glutamic acid as a hydrogen donor. Glutamic-acid dehydrogenase was found to be a constitutive enzyme, whereas the dehydrogenases responsible for the breakdown of glucose had to be formed adaptively. Glutamic acid was superior to glucose as a hydrogen donor only in the first step of the nitrate reduction. Since this step limits the denitrification rate, the presence of amino acids stimulates the entire denitrification process. It was concluded that also denitrification rate in soil by *Ps. aeruginosa* is more readily stimulated by these compounds than by carbohydrates.

When root systems of pea plants were used as a source of hydrogen donors, nitrite was formed as an intermediate product in denitrification by washed cells of *Ps. aeruginosa.* This was assumed to depend on the presence of growing cells, since in separate experiments nitrite accumulation was only found in growing cultures.

Chapter X

The effect of different hydrogen donors on the dissimilatory nitrate reduction by *B. licheniformis* and *B. cereus*

A. Introduction

In chapter VII, it was shown that *B. cereus* was not involved in the denitrification process in grassland soils, although it was the most numerous organism with dissimilatory nitrate-reducing capacity in these soils under normal field conditions. *B. licheniformis* and other *Bacillus*-species (except *B. macerans*) also appeared to be not involved in the process. As contrasted to the species of the *Ps. aeruginosa*-group, the *Bacillus*-species (except *B. macerans*) were unable to produce gas from nitrate when supplied with root excretions as the sole source of hydrogen donors during denitrification. The investigation of the nutritional requirements of the organisms of the *Bacillus-*

group revealed that growth under anaerobic conditions was not always dependent on the presence of nitrate in the medium (chapter VIII). From the agricultural point of view, however, it was important to know, whether or not nitrate can be reduced by these organisms to gaseous products under the conditions prevailing in the soil, irrespective its essentiality for growth under anaerobic conditions.

To explain the ineffectiviness of the *Bacillus*-species in soil denitrification, the influence of nutritional conditions on their dissimilatory nitrate reduction was investigated. The results obtained are reported in this chapter. Attention has been paid mainly to *B. licheniformis;* only a few experiments with *B. cereus* were included.

From the data presented in Table 27 it can be seen that both *Bacillus*-species required amino acids for anaerobic growth. The other species of the *B. licheniformis*-group were not dependent on amino acids, but their growth rate was stimulated by these compounds (Table 28). However, the rate of gas production of these organisms was not affected, in contrast with species of the *Ps. aeruginosa*-group, in which both rate of growth and gas production were stimulated by amino acids. Therefore, the influence of amino acids on dissimilatory nitrate reduction by *B. licheniformis* and *B. cereus* was a point of special interest.

In chapter VIII it was shown that *B. licheniformis* was capable of growing under aerobic conditions in the basal medium with glucose as the carbon source and nitrate as the nitrogen source (Table 27); in the absence of oxygen amino acids were required, an observation in keeping with the results of VERHOEVEN (115) and VALERA and ALEXANDER (113). The other bacteria of the *B. licheniformis*-group behaved differently. To explain the response of *B. licheniformis* to amino acids under anaerobic conditions, use may be made of results obtained by GARY and BARD (39) with the closely related species *B. subtilis* which is aerobic and unable to grow under anerobic conditions in the presence of nitrate. The strain of *B. subtilis* tested by the above authors dissimilated glucose via the hexose-monophosphate pathway and possessed no fermentative capacity when grown in a mineral medium supplemented with glucose. On the contraty, cells grown in a complex medium dissimilated glucose via the Embden-Meyerhof scheme. The latter cells also fermented glucose under anaerobic conditions, although no growth occurred. It appeared that particularly amino acids were responsible for the formation of the enzymes required for the fermentative pathway. Furthermore, it was shown that cells grown in the complex medium, produced more carbon dioxide during the anaerobic breakdown of glucose when supplied with nitrate than without added nitrate. If *B. licheniformis* behaved similarly, amino acids would be required by this species to enable the formation of the enzymes of the Embden-Meyerhof system. In addition, it could be assumed that nitrate under anaerobic conditions is unable to effect the dissimilation of glucose via the gluconate pathway in this organism, but only has a function coupled with the Embden-Meyerhof system.

Aside from the earlier investigations of BEYERINCK and MINKMAN (9) and KLAESER (62), the denitrification by *B. licheniformis* was studied by VERHOEVEN (115). It was shown that in a peptone-containing medium, with glucose, glycerol or lactate as a carbon source, nitrate and nitrite were reduced to nitrous oxide, gaseous nitrogen or ammonia. With some strains a considerable

part of the added nitrate was not accounted for. More recently, the dissimilatory nitrate reduction in cell-free extracts was studied by NAJJAR and ALLEN (75) and by TANIGUCHI *cs.* (109) using strains of *B. subtilis* and *B. pumilus*, respectively. These organisms were recorded as producing nitrogen gas, nitrous oxide and nitric oxide from nitrate (75) and ammonia from nitrite (109). Since *B. subtilis* is unable to produce gaseous products from nitrite (115) and *B. pumilus* cannot reduce this compound either assimilatorily or dissimilatorily, it is very likely that these investigators have dealt with strains of the related *B. licheniformis*.

The breakdown of glucose under anaerobic conditions by *B. licheniformis* was studied by BLACKWOOD *cs.* (11). Lactic acid, 2,3-butanediol, glycerol, ethanol and carbon dioxide, all in varying quantities, were the most abundant products formed. Some strains of this organism did not produce glycerol but formed lactic acid almost exclusively. The organism closely resembles *B. subtilis* in this latter characteristic (39).

Practically no data are available on dissimilatory nitrate reduction by *B. cereus*. HENNEBERG (51) isolated a spore-forming, aerobic bacterium which was able to reduce nitrate to nitrogen dioxide under anaerobic conditions. VERHOEVEN (115), however, was unable to confirm this observation, although he exclusively isolated *B. cereus* from an enrichment culture identical to that of Henneberg, which had an unmistakable smell of nitrogen dioxide. In pure-culture studies with this organism, nitrite was found to be the only reduction product of nitrate, and in no case was any production of nitrogen dioxide noted.

Under anaerobic conditions, in the absence of nitrate, *B. cereus* fermented glucose chiefly to acetylmethylcarbinol, lactic acid, succinic acid, acetic acid and carbon dioxide (88).

B. EXPERIMENTAL

The experiments were performed with *B. licheniformis* str. P 1, kindly supplied by Dr. W. VERHOEVEN, and with *B. cereus* str. 372, isolated from a permanent-grassland soil. The cultures were maintained on peptone-glycerol-nitrate-agar slopes.

1. *Experiments with washed cells*

The effect of different hydrogen donors on the dissimilatory nitrate reduction by these organisms was studied using the washed-cells technique. The cells were grown in Medium 1 (chapter IX). In some experiments the glucose in this medium was replaced by an equal amount of glycerol. The cells were grown and treated similarly to those of *Ps. aeruginosa* (chapter IX). Glucose, glycerol, glutamic acid and vitamin-free casamino acids were used as hydrogen donors, nitrate and nitrite as hydrogen acceptors. *B. licheniformis* was incubated at 37°C and *B. cereus* at 28°C.

Glutamic acid was estimated quantitatively using glutamic acid decarboxylase, as described by GALE (35). Glucose was determined according to the anthron method of TREVELYAN and HARRISON (111). Since glucose, in a sulphuric acid-water mixture, is oxidized by nitrate, the latter compound was removed by reduction prior to analysis. One ml of a 50 per cent H_2SO_4-solution and 50 mg of reduced iron powder were added to 2 ml of glucose solution. The mixture was heated on a boiling water bath until the iron powder had disappeared. Subsequently 1 ml of the solution was mixed with 5 ml of anthron solution (0.2 g anthron per 100 ml of 70 per cent H_2SO_4). The mixture was heated on a boiling water bath for 10 minutes and then rapidly cooled. Finally glucose was determined colorimetrically with a BECKMAN DV spectrophotometer at 620 mμ.

Carbon dioxide was estimated by means of the WARBURG technique. Glycerol and lactic acid were estimated qualitatively by two dimensional paper chromatography using methods of HOUGH (52), and HEITFUSS and WOLFGANG (50), respectively.

2. *Experiments with growing cultures*

The aerobic and anaerobic growth requirements were ascertained in Medium C (with $(NH_4)_2SO_4$ as the nitrogen source) and Medium A (with amino acids as the nitrogen source), see chapter VIII. Test tubes, 18 mm in diameter, were filled with 5 ml (aerobic growth) and 10 ml (anaerobic growth) of the media. Half of the tubes containing 10 ml of medium were supplemented with a nitrate solution making the final concentration 1 per cent. To test aerobic growth, the tubes were placed in a slanting position. For anaerobic growth they were capped with melted vaspar. *B. licheniformis* was incubated at 37 °C and *B. cereus* at 28 °C. The growth was estimated qualitatively.

For the quantitative estimation of the growth of *B. licheniformis* under anaerobic conditions in the presence or absence of nitrate, an experiment was performed in Medium 1 (chapter IX) supplemented with glucose. The cells were separated from the medium by centrifugation, washed twice with distilled water, dried for 24 hours at 105 °C and weighed. The number of viable cells was determined by plating on peptone-glycerol-nitrate-agar. Gas production was estimated by means of a gas burette, upon removal of carbon dioxide.

C. Results and Discussion

1. *Effect of various hydrogen donors on dissimilatory nitrate reduction by B. licheniformis*

In a preliminary experiment on the dissimilatory nitrate reduction by *B. licheniformis* str. P 1, no evolution of gaseous nitrogenous products was observed when washed cell suspensions were supplied with different hydrogen donors. With glucose as hydrogen source, nitrate was mainly reduced to nitrite. Some ammonia was also formed (Table 31). The added nitrate nitrogen

Table 31. Products formed during the dissimilatory nitrate reduction by washed cells of *B. licheniformis* str. P 1 (12 mg dry matter per ml), grown in Medium 1 supplemented with glucose; the cell suspension was supplied with glucose as a hydrogen donor.

Time (minutes)	Nitrogen recovered in μ mole per ml				Glucose μ mole per ml
	NO_3^-	NO_2^-	NH_4^+ [1]	Total	
0	7.64	0.00	0.00	7.64	2.39
45	7.00	0.32	0.25	7.57	2.17
90	5.89	0.81	0.64	7.34	1.78
130	4.97	1.37	1.07	7.41	1.22
195	3.78	2.41	1.21	7.41	0.61
225	2.93	3.09	1.29	7.31	0.00

[1] Values after subtracting the ammonia formed by cells which received no nitrate.

was nearly completely recovered at the end of the experiment. The production of ammonia was irregular, however. Moreover, nitrite could not be used as a hydrogen acceptor by the cells. Therefore, although closed balances were obtained, it is not certain that the ammonia was originating from nitrate; its occurrence may have been the result of the breakdown of cell components under the influence of nitrate, accompagnied by an incomplete recovery of an intermediary reduction product.

In a subsequent experiment the effect of various hydrogen donors on the reduction of nitrate to nitrite was investigated (Table 32). It will be seen that nitrate was reduced only when glucose was present in the suspension. In the presence of glutamic acid or a mixture of amino acids, nitrite production was not effected. A mixture of glucose and amino acids gave considerably higher

amounts of nitrite than the separate substrates. Glycerol and vitamin-free casamino acids were also not used as hydrogen donors under the experimental conditions.

TABLE 32. The effect of various hydrogen donors on the dissimilatory reduction of nitrate (6 μ mole per ml) to nitrite by washed cells of *B. licheniformis* str. P 1, grown in Medium 1 supplemented with glucose.

Hydrogen donor	μ mole per ml	μ mole nitrite recovered per ml Time (minutes)		
		45	110	190
Control, no H-donor		0.07	0.10	0.15
Glycerol	2.25	0.08	0.10	0.16
Glucose	2.40	0.15	0.44	0.94
Glutamic acid	4.75	0.07	0.09	0.15
Glucose + glutamic acid	2.40 + 4.75	0.17	0.99	2.19
Casamino acids	4.50	0.07	0.10	0.15
Glucose + casamino acids	2.40 + 4.50	0.18	1.02	2.08

2. *The uptake of amino acids by B. licheniformis*

To explain the negative results obtained with amino acids as hydrogen donors and the stimulative effect of these compounds on nitrite production when combined with glucose, the suggestion was made that the uptake of amino acids was coupled with the dissimilatory activity of the cells. GALE (36) found such a behaviour in *Streptococcus*-species, when grown in a medium deficient in amino acids. Glutamic acid was taken up by these species only in the presence of glucose; lysine, however, entered the cell mainly by diffusion, and its uptake was not affected by glucose.

The above hypothesis was tested by estimating the uptake of glutamic acid under anaerobic conditions in the presence or absence of glucose (Table 33).

TABLE 33. The uptake of glutamic acid under anaerobic conditions in the presence or absence of glucose by washed cells of *B. licheniformis* str. P 1, grown in Medium 1 supplemented with glucose.

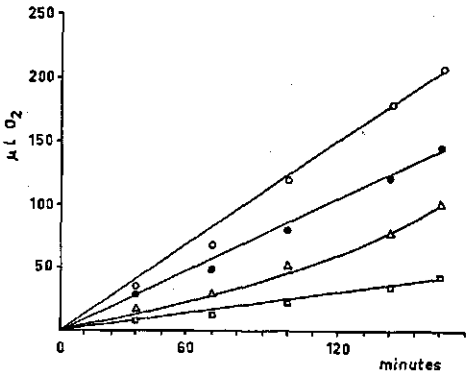
Hydrogen donor	Time (minutes)	Glutamic acid μ mole per ml
Glucose	0	0.21
	180	0.23
Glutamic acid	0	4.06
	180	4.19
Glucose + glutamic acid	0	4.09
	180	2.70

It will be seen that, under the experimental conditions the presence of glucose was required for the uptake of glutamic acid. The same results, which are in accordance with those obtained with vitamin-free casamino acids in the presence and absence of glucose (Table 32), were found with a number of other amino acids. Under aerobic conditions, however, the same cells were able to take up glutamic acid in the absence of glucose (Fig. 12). No attempts were made to investigate the discrepancy between the uptake of amino acids under

FIG. 12.

Oxygen uptake by washed cells of *B. licheniformis* str. P 1, grown under anaerobic conditions in Medium 1 supplemented with glucose; the cell suspension was supplied with different hydrogen donors.

□—□ endogeneous respiration;
△—△ 4.75 μ mole glutamic acid per ml;
●—● 2.45 μ mole glucose per ml;
o—o 4.75 μ mole glutamic acid + 2.45 μ mole glucose per ml.



anaerobic and aerobic conditions. An experiment was carried out, however, to see if *B. licheniformis* was able to grow under anaerobic conditions in a medium containing only amino acids as a carbon source (Table 34). For anaerobic growth, the presence of both glucose and amino acids were required.

TABLE 34. Growth of *B. licheniformis* str. P 1 and *B. cereus* str. 372 under aerobic and anaerobic conditions in the presence or absence of glucose, glycerol, amino acids and nitrate.

Medium	Species	Aerobic			Anaerobic					
		Glucose	Glycerol	Amino acids	Glucose		Glycerol		Amino acids	
					—	NO_3^-	—	NO_3^-	—	NO_3^-
C (mineral)	*B. licheniformis*	+	+	+	—	—	—	—	—	—
	B. cereus	—	—	+	—	—	—	—	—	—
A (amino acids)	*B. licheniformis*	+	+	+	+	+	—	+	—	—
	B. cereus	+	+	+	+	+	—	+	—	—

— No growth + Positive growth.

With glycerol as the carbon source, under anaerobic conditions, nitrate was needed in addition. The presence of the separate compounds enabled growth under aerobic conditions only. These results which are in accordance with the data presented in Tables 27, 29 and 33, indicate that, under anaerobic conditions, the growth requirements of *B. licheniformis* were more complex than those of *Ps. aeruginosa*. This provides further support for the hypothesis that *B. licheniformis* can be easily eliminated in competition with species of the *Pseudomonas*-group during denitrification in the soil.

3. *Glucose metabolism during dissimilatory nitrate reduction by B. licheniformis*

By investigating the effect of the glucose concentration on nitrate reduction, it could be shown that the glucose consumption was largely independent of this reduction (Table 35). With increasing concentrations relatively more glucose was consumed by a mechanism not coupled with the dissimilatory reduction of nitrate.

TABLE 35. The effect of the glucose concentration on the reduction of 6 μ mole nitrate per ml by washed cells of *B. licheniformis* str. P 1, grown in Medium 1, supplemented with glucose.

Initial glucose μ mole per ml	Glucose consumption μ mole per ml	Nitrite production μ mole per ml	μ mole nitrite formed per μ mole glucose consumed
0.26	0.26	0.48	1.88
0.77	0.77	1.00	1.31
1.53	1.53	1.51	0.99
2.39	2.39	1.96	0.82
5.17	4.70	2.31	0.49

In the absence of nitrate the main products formed from glucose by washed cells grown in Medium 1 were lactic acid, glycerol and acetylmethylcarbinol. When nitrate was added, less lactic acid and glycerol were produced, although both compounds were still present when the cells were supplied with 2.39 μ mole glucose (qualitative estimations). At this glucose concentration, 0.75 μ mole carbon dioxide was produced in the absence of nitrate, and 2 μ mole in its presence. These data suggest that nitrate, under the experimental conditions, had a similar function as observed by VERHOEVEN in *E. coli* (117), growing anaerobically in the presence of this compound. This species produced less succinic acid, lactic acid and ethyl alcohol and more acetic acid and carbon dioxide from glucose in the presence of nitrate than in its absence. Apparently part of the formed DPNH is utilized by *B. licheniformis* and *E. coli* to reduce nitrate instead of dihydroxyacetone, pyruvic acid and acetaldehyde.

4. *Experiments with B. cereus str. 372*

When supplied with glucose or glutamic acid as a hydrogen donor, washed cells of this organism, grown in Medium 1, supplemented with glucose, were unable to reduce nitrate to gaseous compounds. The formation of nitrite, a reduction which was accomplished by *B. licheniformis* under the same conditions, was also neglegible (Table 36). Both glucose and glutamic acid were

TABLE 36. The effect of glucose and glutamic acid on the dissimilatory nitrate reduction by washed cells of *B. cereus* str. 372, grown in Medium 1, supplemented with glucose.

Glucose (initial) μ mole/ml	Glutamic acid (initial) μ mole/ml	Glucose (final μ mole/ml	Glutamic acid (final) μ mole/ml	Nitrate (initial) μ mole/ml	Nitrate (final) μ mole/ml	Nitrite (final) μ mole/ml
2.39	0.19	0	0.24	6.00	5.95	0.15
0	4.20	0	1.06	6.00	5.82	0.16
2.39	4.20	0	1.30	6.00	5.87	0.15
0	0.20	0	0.34	6.00	5.92	0.10

taken up under anaerobic conditions when added separately, in contrast with *B. licheniformis* which required glucose for the uptake of glutamic acid (Table 33). However, under these conditions, growth of *B. cereus* occurred only in the presence of both glucose and glutamic acid (Table 34).

5. *The effect of glucose and glycerol on nitrate-reductase activity*

In Table 29 it is shown that nitrate was required by both *B. licheniformis* and *B. cereus* for growth under anaerobic conditions using glycerol as a carbon

source. In the present experiment, the effect of this substrate on nitrate reduction by washed cells of both species, grown in Medium 1 supplemented with glycerol, was investigated. Although no reduction to gaseous compounds was observed, the nitrate-reductase activity was considerably higher than that of cells grown in Medium 1 supplemented with glucose and tested on glucose as a substrate (Table 37).

TABLE 37. Nitrate-reductase activity (mole nitrite produced per mg cell-N per hour) of washed cells of *B. licheniformis* str. P 1 and *B. cereus* str. 372, grown in Medium 1 supplemented with glucose and glycerol, and tested on glucose and glycerol, respectively.

Species	Substrate	Nitrate-reductase activity
B. licheniformis	glucose	0.5
	glycerol	28.0
B. cereus	glucose	0.06
	glycerol	21.0

It is unlikely that amino acids and glycerol or lactate will be available in sufficient amounts in soil to enable any appreciable dissimilatory nitrate reduction by *B. licheniformis* and *B. cereus,* even when these organisms are not eliminated by other denitrifying bacteria. On the contrary, *Pseudomonas*-species are better adapted to perform denitrification in the soil. The nutritional requirements of *B. macerans* and *Achromobacter* str. 181 were not sufficiently investigated to provide an explanation of the phenomenon of both these species being involved in soil dentrification.

In cans containing nitrate-cured ham, an ample supply of amino acids and glycerol derived from fats is available, while non-sporeforming denitrifying bacteria are killed during the conservation procedure. Therefore, it is not surprising that *B. licheniformis* was isolated when VERHOEVEN (114) investigated the cause of the swelling of cans containing cured ham.

6. *Gas production in growing cultures*

The results reported in the preceding section disagreed with those of VERHOEVEN (115) in that the formation of nitrous oxide or nitrogen gas was not observed. This was true of washed cells; growing cells of the spore-forming, denitrifying bacteria which were also used by VERHOEVEN, did form gaseous products (Table 29). These results agree with those of DR. F. D. COOK, Ottawa (personal communication) who also found that gaseous reduction products from nitrate were formed in growing cultures of *B. licheniformis,* but not by washed cells. Attempts made by the present author to explain the different behaviour of growing cultures and washed cells were unsuccesful. However, it may be worthwhile to record some observations.

Although it is firmly established that nitrite is the first reduction product of nitrate, the mechanisms beyond the nitrite stage are still much in need of clarification. Some evidence was obtained that nitric oxide, or a compound with which it equilibrates, is an intermediate in both assimilatory and dissimilatory nitrate reduction (32). The role of nitrous oxide is especially obscure. Apparently it is not involved as an intermediate compound (97), as was suggested by KLUYVER and VERHOEVEN (63, 115). Several workers have reported that nitrous oxide can be reduced to gaseous nitrogen or ammonia (115, 97, 77); for this reaction the formation of an adaptive enzyme is necessary. Re-

cently, FEWSON and NICHOLAS (34) suggested that nitrous oxide may be formed non-enzymatically when a compound at the nitroxyl level accumulates. Although this suggestion was not supported by any experimental evidence, it may explain to a large extent the results obtained with growing cultures of *B. licheniformis*.

In the experiments of Verhoeven with growing cultures of *B. licheniformis*, gas production usually started after some lag period and continued at a linear rate after nitrate and nitrite had been completely consumed. In the earlier stages of growth, nitrous oxide was the main product formed; the evolution of nitrogen gas usually began at a more advanced stage. Thus, VERHOEVEN supposed that nitrous oxide accumulated as an intermediate product in nitrate reduction and gave rise to the formation of gaseous nitrogen. As to the enzymatic nature of the nitrous-oxide producing reaction, no data were presented. Moreover, the methods remained obscure by which VERHOEVEN succeeded in obtaining more or less closed nitrogen balances in the presence of paraffin oil, a substance known to absorb nitrous oxide to such a high degree that it is used to estimate this compound quantitatively.

In the present investigation, it was observed that the production of gas from nitrate continued long after the growth of *B. licheniformis* had ceased. During this period the amount of cell material, as well as the number of viable cells, decreased (Table 38). Hence, it is unlikely that gas production was connected

TABLE 38. Dry-matter production and numbers of viable cells of *B. licheniformis* str. P 1 grown in Medium L, supplemented with 1 per cent glucose in the presence and absence of nitrate.

Time (days)	No nitrate; dry matter mg/100 ml	1 per cent nitrate		
		Dry matter mg/100 ml	Number of viable cells	Gas production ml/100 ml
1	26.0	13.7	8.10^6	4
2	26.1	13.1	3.10^5	7
3	26.9	12.7	2.10^3	14
4	27.0	8.7	1.10^2	31
9	28.0	8.4	0	49

with the active metabolism of the cells, since the carbon source was also exhausted. This result may explain the fact that no gas production was observed with washed cells exhibiting an active metabolism when supplied with a carbon source.

The low yields of cell material in the presence of nitrate (Table 38) were largely due to the accumulation of nitrite which had a detrimental effect on growth, as was shown in a separate experiment. When the nitrate in Medium 1, supplemented with glucose, was replaced by an equivalent amount of nitrite, only scant growth occurred, although a considerable amount of gas was produced.

D. SUMMARY

No gaseous reduction products were formed from nitrate by washed cells of *B. licheniformis* during the dissimilatory nitrate reduction in the presence of various hydrogen donors. Only nitrite and ammonia were detected as reaction products. Under anaerobic conditions, the uptake of amino acids ap-

peared to be coupled with glucose consumption. No direct relationship was observed between the uptake of glucose and dissimilatory nitrate reduction. Washed cells of *B. cereus* grown in a glucose-containing complex medium were unable to reduce nitrate dissimilatorily. The nitrate-reductase activity of washed cells of *B. licheniformis* and *B. cereus*, grown in a medium containing glycerol as a carbon source, was found to be considerably higher than that of cells grown in the presence of glucose. It was suggested that the conditions under which these organisms possess an optimal nitrate-reducing activity will seldom be found in the soil. Evidence was obtained concerning the non-enzymatic nature of the formation of gaseous reduction products from nitrate in growing cultures of *B. licheniformis*.

CHAPTER XI

THE REDUCTION OF NITRATE TO AMMONIA BY DENITRIFYING BACTERIA

A. INTRODUCTION

During the experiments described in chapter IV, it was observed that, after adding tagged nitrate to sods of a permanent-grassland soil, containing living roots, considerable quantities of the tracer were found in the organic-matter fraction of the soil. Direct incorporation of nitrate nitrogen into the soil organic matter being unlikely (chapter IV), to explain this result the following mechanisms were considered. Firstly, the nitrate might have been taken up by the plants and excreted afterwards as organic nitrogenous compounds. Secondly, the reduction of nitrate to ammonia, followed by incorporation of the latter into the soil organic matter might be the case. In order to pursue this problem, one must know to what extent reduction of nitrate to the ammonium level occurs in the soil. The question is also important from the agricultural point of view, since ammonia, in contrast with gaseous reduction products derived from nitrate, remains in the soil.

Although an accumulation of ammonia during the denitrification process in soil has often been observed, experiments with labeled nitrate have given negative results, i.e. the ammonium fractions did not contain appreciable amounts of label (19, 41, 79, 126). Therefore, it seems likely that the ammonia had originated from deaminating reactions. This hypothesis is given support by results of BROADBENT and STOJANOVIC (19) who observed that, during the occurrence of denitrification in the soil as compared with aerobic conditions, the carbon-dioxide production but not the ammonification was retarded. Moreover, in the experiments with washed cells of *Ps. aeruginosa*, it was found that a rapid hydrogen transfer to nitrate occurred during the deamination of glutamic acid.

On the other hand, there are many reports of the ability of pure cultures of microorganisms to reduce nitrate and other oxidized forms of nitrogen to ammonia (76, 109, 115, 125). VERHOEVEN (115) showed that, particularly in the cases of *B. licheniformis* and *B. subtilis*, large amounts of ammonia may be produced under certain conditions. This led Verhoeven to the conclusion that the reduction of nitrate to ammonia in the soil would be of practical importance, especially since *B. subtilis* is a common soil organism. Till now it

has not been possible to support this statement with experimental evidence. Nevertheless, the question arises of why some common soil bacteria, which in pure-culture studies reduces nitrate to ammonia, are unable to perform this reaction during denitrification in soil. Perhaps the organisms which are able to bring about this reduction, are not involved in the dissimilatory reduction of nitrate in the soil. Doubtlessly, this explanation is true of *B. licheniformis* (chapter X). Nevertheless, when this organism was added to sterile root systems of pea plants in vermiculite supplied with nitrate, formation of ammonia was observed (Table 26). It was unknown, however, whether this ammonia was derived from nitrate, or from amino acids excreted by pea roots.

Some experiments were carried out with grassland sods in order to obtain more information concerning a possible reduction of nitrate to ammonia in permanent-grassland soils, and the contribution of different species of denitrifying organisms to the process.

B. Results

1. *The formation of ammonia in grassland sods*

The capacity of the permanent-grassland sods, derived from sandy soil (see chapter III), to reduce nitrate to ammonia, was investigated by incubating sods with tagged nitrate nitrogen. Sods, 4.5 cm in diameter and 3 cm thick, contained in plastic baskets, were incubated in large Warburg vessels at 25°C. The air inside the vessels was replaced by nitrogen gas. One series of sods received 20 mg nitrate nitrogen of which 4 mg were tagged; a second series received no nitrogen. Gas production was followed manometrically. In replicate sods the nitrate, nitrite and ammonium fractions were estimated after different times of incubation. The percentage label of the ammonium fraction was determined with the aid of a Metropolitan Vickers mass spectrometer after conversion of the ammonia into gaseous nitrogen. The results of this experiment are presented in Table 39. It will be seen that, under the experimental conditions, the formation of ammonia from nitrate was neglegible

Table 39. The dissimilatory reduction of 20 mg partially labeled nitrate nitrogen to ammonia by sods of a permanent-grassland soil.

Nitrogen addition	Incubation time (hours)	Mg nitrogen recovered per sod						
		NO_3^-	NO_2^-	NH_4^+			N_2+N_2O	Total
				Total	% excess ^{15}N	Fert. N		
20 mg NO_3–N	16	11.4	0.6	1.75	0.42	0.04	2.6	16.4
	28	9.5	0.4	7.95	0.36	0.14	3.6	20.5
	40	8.6	0.4	2.50	0.44	0.05	5.2	16.7
	52	8.5	0.1	2.00	0.45	0.04	5.6	16.2
	76	1.4	0.8	7.20	0.34	0.12	13.8	23.2
	100	0	0	6.90	0.67	0.27	19.2	26.1
no nitrogen	16	0	0	1.10	–	–	0	1.1
	28	0	0	1.70	–	–	0	1.7
	40	0	0	2.10	–	–	0	2.1
	52	0	0	1.80	–	–	0	1.8
	76	0	0	1.50	–	–	0	1.5
	100	0	0	6.40?	–	–	0	6.4

and hardly exceeded the 1 per cent level at all stages of the denitrification process. Nevertheless, the total amount of ammonia in the sods was clearly higher upon the addition of nitrate. When the experiment was repeated with unlabeled nitrate, similar results were obtained. It may be concluded that the denitrification process is attended by an enlarged desamination activity. The outcome of these experiments is in agreement with the results of other investigators (19, 41, 79, 84, 126), i.e. that during denitrification in soil the reduction of nitrate to ammonia is of no practical importance. Only in the case of soils containing considerable amounts of ferrous ions is a chemical reduction possible.

2. *Experiments with root systems of pea plants inoculated with B. licheniformis*

In a second experiment, the extent to which *B. licheniformis* was capable of reducing nitrate to ammonia under conditions prevailing during denitrification in the rhizosphere, was investigated. Root systems of sterile pea plants were cultivated as described in chapter VI.B.2. When the plants were 12 days old, a suspension of *B. licheniformis* was added along with 20 mg sterile nitrate nitrogen, 4 mg of which were tagged. The filters containing the inoculated root systems, were placed under sterile conditions in Warburg vessels so that gas production could be measured after the air inside the vessels had been replaced with oxygen-free nitrogen gas. At different incubation times the nitrate, nitrite and ammonium contents of replicate filters were estimated (Table 40).

TABLE 40. The reduction of 20 mg partially labeled nitrate nitrogen by *B. licheniformis* str. P 1 in the presence of hydrogen donors supplied by sterile pea plants.

Time (hours)	Nitrogen recovered in mg N						
	NO_3^-	NO_2^-	NH_4^+			$N_2 + N_2O$	Total
			Total	% excess ^{15}N	Fert. N		
0	19.9	0.0	0.1	–	–	0	20.0
30	15.0	3.2	1.4	2.58	0.18	0	19.6
60	8.2	9.1	1.9	2.63	0.25	0	19.2
100	7.4	9.3	2.5	2.45	0.31	0	19.2

It appeared that approximately 1.5 per cent of the added nitrate was reduced to ammonia. The percentage excess ^{15}N in the ammonia was higher than that in the experiment recorded in Table 39. This indicates that more nitrate was reduced to ammonia by *B. licheniformis* than by the mixed denitrifying population of permanent-grassland sods. Therefore, it is unlikely that this population consisted mainly of *B. licheniformis;* if this had been so, the percentage excess ^{15}N in the ammonium fraction of the grassland sods would have been higher. This result is in agreement with those of chapters VII, VIII and X in which it was shown that *B. licheniformis* is not involved in soil denitrification. The data of Table 40 show that also in this experiment the greater part of the ammonia originated from deamination reactions. Nitrate was reduced mainly to nitrite, while gaseous products, in contrast with the experiment with sods (Table 39), were not observed. These results which corroberate with the data given in Table 26, clearly demonstrate that denitrifying organisms other than *B. licheniformis* are present in the sods.

3. *Formation of ammonia from nitrate by B. licheniformis and B. subtilis in culture media.*

Since hardly any nitrate was reduced to ammonia during denitrification with sterile pea plants, it was supposed that the conditions under which pure cultures are able to carry out this process, were not encountered in that experiment. To test this hypothesis, an attempt was made to elucidate these conditions in some preliminary experiments with *B. licheniformis* and *B. subtilis* which are common soil organisms. VERHOEVEN showed that these species produce large quantities of ammonia when grown in a mineral medium under „semi-aerobic" conditions.

a. Experiments in Erlenmeyer vessels with a shallow layer of nutrient medium. In an experiment with *B. licheniformis*, the validity of VERHOEVEN's observations was checked. *B. licheniformis* str. P 1 was grown under the conditions described by VERHOEVEN (115). The organism was cultivated in 300 ml Erlenmeyer flasks, containing 100 ml of a medium consisting of: glucose, 10 g; K_2HPO_4, 1 g; $MgSO_4.7\,H_2O$, 0.5 g; KNO_3, 10 g; tap water, 1 litre. The depth of the medium was about 35 mm. The Erlenmeyer flasks were plugged with a rubber stopper in which glass inlet and outlet tubes were inserted for a continuous air renewal of the atmosphere above the medium. The entering air first passed through two washing-bottles containing sulphuric acid to remove ammonia, and then through a washing-bottle containing distilled water in order to saturate the air with moisture. On leaving the culture flask, the air passed through a washing-bottle containing boric acid in order to trap evolved ammonia. The rate of the air current passing through the flask was about 60 ml per minute. After inoculation, the flasks were incubated at 37°C in a water bath. At different times of incubation, the nitrate, nitrite, ammonia and organic-nitrogen contents of replicate flasks were estimated. The number of viable cells was counted by plating a dilution series of the medium on peptone-glycerol-nitrate-agar. The result of this experiment is shown in Table 41.

TABLE 41. The fate of nitrate nitrogen in a shallow-layer culture of *B. licheniformis* str. P 1 with surface aeration (mineral medium with glucose as the carbon source).

Time (days)	Nitrogen recovered in mg N per 100 ml medium						Number of cells per ml
	NO_3^-	NO_2^-	NH_4^+	Soluble org. N	Bact. N	Total	
0	133.9	0.0	0.0	0.0	0.0	133.9	8.10^2
3	107.0	16.5	1.7	0.0	4.8	130.0	6.10^4
7	91.2	5.0	24.9	0.6	13.4	135.1	3.10^7
10	84.2	7.0	32.6	1.0	8.2	133.0	2.10^7
13	77.6	8.0	38.4	1.8	8.7	134.5	2.10^7
18	69.1	5.0	48.1	2.0	10.2	134.4	3.10^7

Although at a lower rate than in the experiments of VERHOEVEN, considerable quantities of ammonia were produced, mostly at a stage when the number of viable cells was no longer increasing. Similar results were obtained with some other strains of *B. licheniformis* and with *B. subtilis* str. Marburg. No gaseous nitrogenous reduction products were observed during the dissimilatory reduction of nitrate. These results were entirely different from those obtained with growing cultures of these bacteria in complex media under anaerobic conditions

when nitrate was reduced chiefly to nitrogen gas and nitrous oxide. The occurrence of different reduction products under semi-aerobic and anaerobic conditions was explained by VERHOEVEN (115) by assuming different degrees of susceptibility to oxygen on the part of the enzymes involved in the production of ammonia and gaseous reduction products, respectively. Such an explanation is unsatisfactory, as no data were presented concerning the degree of anaerobiosis in the shallow layers of the culture medium. It may be assumed that local spots of oxygen deficiency will have occurred in the shallow layers, since dissimilatory nitrate reduction is usually only observed at very low levels of dissolved oxygen. Very likely a different mechanism must have underlain the observed differences in reduction products. As shown in chapter VIII, no growth of *B. licheniformis* occurs under anaerobic conditions in a mineral medium supplemented with glucose, conditions used also in the shallow layer experiment. Therefore, the cells in the latter experiment, which exhibited dissimilatory nitrate-reducing capacity, must have developed under aerobic conditions. These cells possessed an assimilatory nitrate-reducing enzyme system, since nitrate was the sole source of nitrogen. The same holds for *B. subtilis* which is unable to grow in the absence of oxygen; nevertheless this organism, like *B. licheniformis* when cultivated in the minimal medium in Erlenmeyer flasks, was able to reduce nitrate dissimilatorily to ammonia. Therefore, it was suggested that the enzymes needed for the dissimilatory formation of ammonia from nitrate developed only when the cells had been grown under aerobic conditions in a minimal medium and subsequently had been submitted to anaerobic conditions.

To explain the behaviour of *B. licheniformis* and *B. subtilis*, the experiments of GARY and BARD (39), mentioned in the preceding chapter, may be recalled. These authors found that cells of *B. subtilis*, grown in a mineral medium supplemented with glucose, dissimilated glucose via the monophosphate pathway; in a complex medium the Embden-Meyerhof system functioned. Since *B. licheniformis* is closely related to *B. subtilis* and presumably behaves similarly, the implication is that the dissimilatory nitrate reduction of both organisms, when grown in a minimal medium, is linked with the hexose-monophosphate pathway of carbohydrate breakdown. In the presence of amino acids, the dissimilatory nitrate reduction of *B. licheniformis* under anaerobic conditions is coupled with the glycolytic pathway. The latter was made plausible in chapter X.

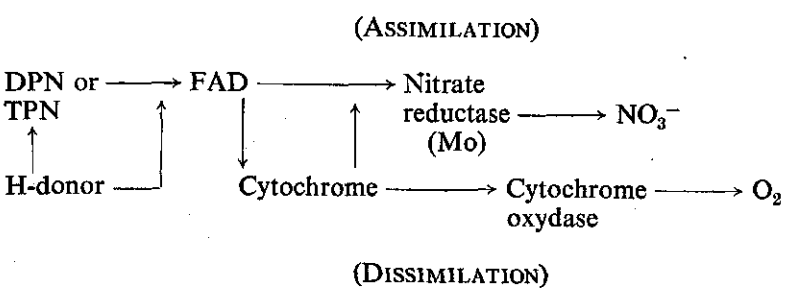
b. Experiments with washed cells of *B. licheniformis* grown aerobically on potassium nitrate and subsequently submitted to anaerobic conditions. To test the hypothesis that dissimilatory ammonia formation from nitrate was accomplished by cells grown aerobically in a minimal medium and subsequently submitted to anaerobic conditions, *B. licheniformis* str. P 1 was grown in the mineral medium under vigorous aeration. During the growth of the organism, no accumulation of nitrite or ammonia was observed and reduction of nitrate was restricted to that part used for assimilatory purposes. A similar result was obtained by VERHOEVEN (115). When 4 days old, the cells were harvested, washed once with distilled water and once with 0.05 M phosphate salts buffer, pH 6.9. Subsequently, 1 ml cell suspension (containing 4 mg dry matter) was incubated with 2.4 μ mole glucose and 12 μ mole nitrate in Warburg vessels. The air inside the vessels was replaced by oxygen-free nitrogen gas. Since *B. licheniformis* forms no gas from nitrate under such conditions,

carbon-dioxide production could be followed manometrically. Nitrate, nitrite and ammonia were estimated periodically in replicate vessels. The result of this experiment is presented in Table 42. It appears that nitrate was reduced to nitrite and ammonia without an apparent lag. Carbon-dioxide production also started immediately after adding the glucose. About 75 per cent of the glucose was recovered as carbon dioxide, an amount which was considerably higher than that formed anaerobically in complex media. When nitrite instead of nitrate was used as a hydrogen acceptor, ammonia was formed also without any adaptation period.

TABLE 42. Dissimilatory reduction of nitrate to nitrite and ammonia by washed cells of *B. licheniformis* str. P 1, grown aerobically in a mineral medium containing nitrate as a nitrogen source, and suspended in phosphate salts buffer under anaerobic conditions with glucose as a hydrogen donor and nitrate as a hydrogen acceptor.

Time (minutes)	Nitrogen recovery in μ mole per ml			
	NO_3^-	NO_2^-	NH_4^+	Total
0	12.0	0	0.01	12.0
10	11.2	0.5	0.37	12.1
30	9.1	1.7	1.10	11.9
60	5.8	3.4	2.70	11.9
120	0	7.0	5.10	12.1

Since the production of nitrite and ammonia by the aerobically grown cells started without an adaptation period under anaerobic conditions (Table 42), the cells must have already possessed the enzyme system required for dissimilatory nitrate reduction. Alternatively, a separate dissimilatory nitrate-reducing enzyme system may have been present in the cells, the formation of which was not inhibited by oxygen. Obviously the latter system was not operating under aerobic conditions, as no ammonia accumulated during aerobic growth of the cells. In general, molecular oxygen has been reported as reversibly inhibiting action as well as adaptive formation of the dissimilatory enzyme system (63, 84, 85, 96, 99, 114). One can explain this by assuming that cytochromes are involved in dissimilatory nitrate reduction. Under aerobic conditions the reduced state of the cytochromes would be oxidized by cytochrome oxydase in preference to nitrate reductase. However, during nitrate assimilation cytochromes should not be involved. Thus, this process would be influenced by oxygen, (cf. the following scheme drawn up by FEWSON and NICHOLAS (34):



However, it is in no way certain that cytochromes are always involved in nitrate dissimilation. It should be noted that iron-deficient cells of bacterial

species possessing dissimilatory nitrate-reducing activity, still showed this enzyme activity, when grown anaerobically in the presence of nitrate (34). A number of *Clostridium*-species, found to be able to reduce nitrate at the expense of molecular hydrogen, is known to possess no cytochromes (90, 125). Moreover, SATO (98) found that the reduced state of the cytochromes of *B. subtilis* was not affected by the nitrate-reduction process, which indicates that these compounds played no part in the electron transport from substrate to nitrate. These data suggest that, at least in certain microorganisms, cytochromes are not involved during dissimilatory nitrate reduction. Therefore, in these organisms another mechanism exists to effect dissimilatory nitrate reduction. Probably the enzyme system involved in dissimilatory nitrate reduction by aerobically grown cells of *B. licheniformis* is of the latter type. The results obtained with the aerobically grown cells of this species indicate that this enzyme system is identical with, or closely resembles that of the assimilatory one. Further experiments are required to establish its nature definitely.

c. Experiments with washed cells of *B. licheniformis* grown aerobically on ammonium nitrate and subsequently submitted to anaerobic conditions. The close relationship between the assimilatory and dissimilatory nitrate-reducing enzyme systems of *B. licheniformis* str. P 1 was further shown by growing the organism aerobically in a similar mineral medium with glucose as used in the previous experiment, except that potassium nitrate was replaced by ammonium nitrate. When the washed cells were placed under anaerobic conditions in the presence of potassium nitrate using glucose as a hydrogen donor, no formation of ammonia was observed (Fig. 13). Nitrite was produced

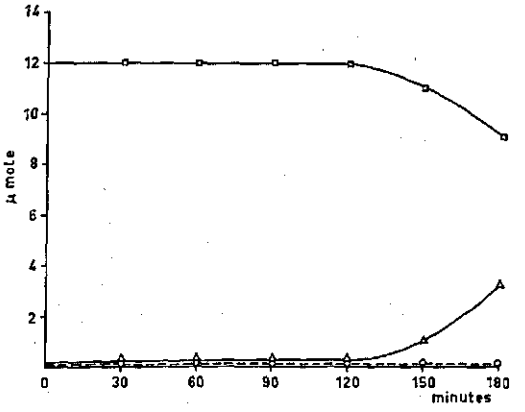


FIG. 13.
Dissimilatory reduction of 12 µ mole nitrate by washed cells of *B. licheniformis* str. P 1, grown aerobically in a mineral medium containing ammonium nitrate as a nitrogen source; 2.4 µ mole glucose per ml was used as a hydrogen donor.
□—□ nitrate (µ mole per ml);
△—△ nitrite (µ mole per ml);
o----o ammonia (µ mole per ml).

as the sole reduction product only after a considerable lag period. This result can be explained by assuming that the presence of ammonium nitrogen had prevented the formation of both the assimilatory and dissimilatory nitrate-reducing enzyme systems, since a similar result was obtained when ammonium sulphate was used as a nitrogen source for the cultivation of the cells.

A repression of the nitrate assimilation by ammonia was also observed with *Ps. denitrificans* and *Ps. fluorescens* (70) and with *M. denitrificans* (23), when these organisms were grown in the presence of both nitrate and ammonia. However, the enzymes involved in the dissimilatory nitrate reduction by anaerobically grown cells of the above species are not affected by ammonia. Data

presented by FEWSON and NICHOLAS (34, Table 3) suggest, that nitrate assimilation in *E. coli* is not repressed by ammonia.

d. Experiments with washed cells of *B. subtilis* str. Marburg, grown aerobically on potassium nitrate and subsequently submitted to anaerobic conditions. That the dissimilatory enzyme system of the bacilli is presumably not entirely identical with the assimilatory one, was indicated in an experiment with washed cells of *B. subtilis* str. Marburg grown aerobically in a mineral medium with nitrate as the nitrogen source. The cells were unable to reduce nitrate to ammonia, when submitted to anaerobic conditions. Nitrite, however, was readily converted into ammonia under these conditions. This result, which indicates that the nitrate-reductase activity of aerobically grown *B. subtilis*-cells was not sufficiently high to enable dissimilatory nitrate reduction, disagrees with that of the shallow layer experiment in which ammonia was found to be produced from nitrate also by this species. It must be stated, however, that the experiment with washed cells lastened only a few hours, whereas the experiment with shallow layer cultures continued for several days.

4. *Application of the results obtained with pure cultures to soil conditions*

The experiments described above were designed to clarify the conditions under which nitrate was reduced by *B. licheniformis* and *B. subtilis* to ammonia. It was shown that the accumulation of this compound observed by VERHOEVEN (115), often used as an argument in favour of the possible occurrence of the process in soil denitrification, only occurs in cells grown aerobically in the absence of ammonium salts. Since ammonium nitrogen is always available due to mineralization of organic compounds, it is unlikely that the required conditions for the reduction of nitrate to ammonia will occur in the soil. Therefore, it must be concluded that the process observed by VERHOEVEN does not contribute to the reduction of nitrate to ammonia in the soil.

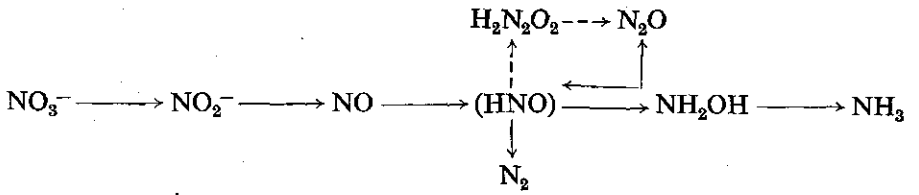
It may be assumed that conditions prevailing in soil upon the addition of nitrate are also not suitable for other organisms (e.g. clostridia, certain strains of *E. coli*) which are able to produce ammonia from nitrate dissimilatorily. In the case of the clostridia which are known to be completely repressed by adding nitrate to various substrates (e.g. submerged patatoes, silage above pH 5; personal communication of MR. G. W. WIERINGA), this is probably due to an unfavourable oxydation-reduction potential.

From the results presented in this section it may be concluded that reduction of nitrate to ammonia by certain denitrifying bacteria is unlikely to proceed in soil. This is due to the fact that the conditions, under which pure cultures of soil bacteria are able to bring about this reduction, do not occur in soil.

C. SOME REMARKS CONCERNING ENZYMATIC NITRATE REDUCTION

The experiments described in section B of this chapter, subscribe to the conception of FEWSON and NICHOLAS (34) concerning a common pathway in nitrate assimilation and dissimilation. The same intermediates appear to be involved in both processess and denitrification may be considered as an adaptation of the assimilatory enzyme system to remove toxic compounds which may accumulate when nitrate instead of oxygen is used as a hydrogen acceptor in respiration. A control mechanism exists by which the activity of the dissimilative enzyme systems is repressed by oxygen. This repression is due

to the competition for electrons between oxygen and nitrate in which the former competes more succesfully. Oxygen has no harmful effect on the enzymes *per se*. In many organisms cytochromes are probably involved in the control mechanism, although the inhibition of nitrate dissimilation by oxygen in *B. licheniformis* and *B. subtilis* cannot be explained in this way. The scheme for nitrate reduction given by FEWSON and NICHOLAS (34) seems to be the best representation of present knowledge:



——→ enzymatic

----→ non enzymatic

No data are available supporting the view that the reduction of nitrous oxide to nitrogen gas proceeds via a compound at the nitroxyl level as suggested in this scheme.

In general, two different types of nitrate reduction are distinguished: nitrate assimilation and nitrate respiration. It should be realized that nitrate is used as a terminal hydrogen acceptor in both cases. During assimilation, the ultimate reduction product, i.e. ammonia, is incorporated into cell material; during dissimilation nitrate is merely used as a hydrogen acceptor, especially, when other more suitable acceptors (like oxygen) are lacking or are not supplied at a sufficient rate. Therefore, each case, in which nitrate is not completely assimilated, and intermediate reduction products like nitrite, nitrous oxide, nitrogen gas or ammonia accumulate in the medium, should be considered as a form of dissimilatory nitrate reduction. Between the production of sometimes small quantities of nitrite, used as a characteristic in determinative bacteriology, and the formation of gaseous products by organisms, which under anaerobic conditions are depending for growth on the presence of nitrate, a scale of different "degrees" of dissimilatory nitrate reduction exists.

The dissimilative reduction of nitrate will not necessarily favour the production of cell material, since the process is not always linked to energy-yielding reactions. Although evidence has been provided for the coupling of phosphorylation to nitrate respiration (80, 108), KESSLER and BUCKER (61) stated that this would not occur when nitrite is used as a hydrogen acceptor.

In the experiments with *B. licheniformis* (chapter X) no increased dry-matter production was observed in anaerobic cultures with glucose as the carbon source owing to the presence of nitrate. Nitrate may even have a harmful effect (Table 38). This effect of nitrate was also observed in shallow-layer cultures. The organism was grown in the minimal medium (described in Section B.3 of this chapter), containing two different quantities of nitrate. After 12 day's incubation, the distribution of the nitrogen and the yield of cell material were estimated (Table 43). It will be seen that the highest yield of cell material was obtained at the lowest nitrate level. This result may be explained by assuming that at the high nitrate concentration a considerable part of the glucose did not serve synthesis of cell material but was used to reduce nitrate,

TABLE 43. The effect of the nitrate concentration on the nitrogen distribution and dry-matter production of *B. licheniformis* str. P 1, grown in shallow layers of a minimal medium, containing nitrate as a nitrogen source.

Initial nitrate mg N/100 ml	Nitrogen recovery in mg N per 100 ml medium					Yield of cell material, mg per 100 ml medium
	NO_3^-	NO_2^-	NH_4^+	Cell N	Total	
114	51.1	0.3	52.0	10	113	200
33	0.0	0.0	17.0	14	31	260

a process which, in this species, is not coupled with energy-yielding reactions. At the low nitrate concentration the glucose was used more efficiently.

In organisms like *Ps. aeruginosa* and *M. denitrificans* in contrast to *B. licheniformis* and *B. subtilis*, the process is undoubtedly attended with ATP-formation. The comparison of dry-matter production of cells of these organisms grown with oxygen or nitrate as terminal hydrogen acceptors, respectively, have yielded conflicting results. Dry-matter production in the presence of nitrate under anaerobic conditions has been reported to be nearly the same (23, 116), lower (30, 31) and distinctly lower (97) than that in the presence of oxygen. These data show that the coupling of dissimilatory nitrate reduction to energy-yielding reactions is a subject still much in need of clarification.

D. SUMMARY

The reduction of nitrate to ammonia during denitrification in soil was investigated by incubating grassland sods with labeled nitrate under anaerobic conditions. Considerable quantities of ammonia were formed, mainly originating from deamination reactions and, only to a small extent, from nitrate. *B. licheniformis*, which may produce high amounts of ammonia from nitrate in pure culture under certain conditions, did not form ammonia from nitrate under conditions prevailing in the rhizosphere of pea plants. In pure-culture studies with this organism, it appeared that cells grown aerobically in a mineral medium with glucose as the carbon source and nitrate as the sole nitrogen source, were able to reduce nitrate and nitrite dissimilatorily to ammonia, when submitted to anaerobic conditions. When ammonia was present in the growth medium, the dissimilatory formation of ammonia from nitrate was completely inhibited. A close relationship was shown to exist between the assimilatory and dissimilatory nitrate-reducing enzyme systems of aerobically grown cells of *B. licheniformis*. It was concluded that the conditions prevailing in the soil are not suitable for an appreciable formation of ammonia by this species.

Some aspects of nitrate reduction, *viz.* common features of assimilatory and dissimilatory nitrate reduction, and the coupling of energy-yielding reactions to dissimilatory nitrate reduction, were briefly discussed.

SUMMARY

The low recovery of fertilizer nitrogen in the herbage of permanent pastures (usually less than 60 per cent) provided the motivation for the present investigation which endeavoured to trace the part of the nitrogen unaccounted for in the tops. Immobilization in plant roots, microorganisms and soil organic matter, leaching, and volatilization may be responsible for the observed losses. The high quantity of nitrogen already present in the sod of permanent-grassland soils prevented to trace the missing part of the fertilizer by conventional methods. Therefore, the majority of the experiments was carried out by applying labeled nitrogen, natural conditions being imitated as closely as possible.

In preliminary experiments (1958) labeled nitrate was supplied to sods of permanent grassland, derived from peat, clay and sandy soil. The sods were placed in pots without holes, in order to prevent leaching; two moisture levels were applied, corresponding to 80 and 95 per cent of the waterholding capacity. After about two months, nitrogen balances of the sods were established by analysing herbage, roots and soil. The nitrogen not accounted for was considered to be volatilized. In all sods tested, a considerable part (15–37%) of the added fertilizer nitrogen was found to be volatilized; the roots and the soil also contained part of the tracer (Table 6). A linear, negative correlation existed in the separate pots between uptake of fertilizer nitrogen in the herbage and the nitrogen not accounted for (Fig. 1). In clay and sandy soil, the influence of the moisture level on the loss of nitrogen was not significant. Only in peaty soil, volatilization had increased at the high moisture level.

An additional experiment with labeled nitrate was carried out to investigate the influence of the cutting frequency of the herbage on nitrogen balances and nitrogen distribution. The cutting frequency was found to have no influence on volatilization; the roots and the soil, however, retained more fertilizer nitrogen upon delaying the first cut (Table 7). There were indications that a downward movement of fertilizer nitrogen from the herbage to the roots and the soil may occur (Table 8).

Similar experiments were carried out in 1959 with sods of permanent grassland (at 60% of the waterholding capacity) which received tagged nitrate and ammonia. Nitrogen balances were established as soon as the mineral nitrogen had disappeared from the soil (T_0). A comparison was made with nitrogen balances of sods which were analysed one month later (T_1). Losses of mineral nitrogen occurred both after a nitrate dressing (20 per cent loss) and an ammonium dressing (9 per cent loss), see Table 11. Between T_0 and T_1 no further losses occurred, indicating that no nitrogen volatilized from the ageing herbage. In case of nitrate fertilization, the losses were ascribed to denitrification.

In subsequent experiments, nitrogen balances in sods with killed roots were established in order to elucidate the effect of living plants on denitrification. Mineralization of soil nitrogen and immobilization of fertilizer nitrogen were also studied. Mineralization rate in sods derived from sandy soil was low; it could be highly increased, however, by mixing the sods thoroughly (Table 12). Nitrification in the sods was retarded as compared with that in the subsoil. When labeled nitrate and ammonium nitrogen were added to the sods with killed roots, nitrogen losses were found to be considerably lower than in case of sods with living roots (9 per cent upon application of nitrate and no losses

upon application of ammonia, see Table 14). It was concluded that living plants have a stimulative effect on denitrification. In addition it appeared that, although a considerable amount of ^{15}N derived from added fertilizer nitrogen was incorporated in the soil organic matter, no gains in total organic soil nitrogen by surplus immobilization of fertilizer nitrogen occurred, incorporation being due to the exchange of fertilizer nitrogen and mineralized soil nitrogen followed by microbiological immobilization ("Turnover", see also page 8). Nitrate was involved only to a small extent in the nitrogen turnover. This could be explained in experiments with pure cultures of soil microorganisms; it was shown that ammonia was preferred to nitrate by these organisms as a nitrogen source (Table 15). Indications were obtained that, with nitrate being used as a fertilizer in case of sods with living roots, part of the nitrogen was incorporated in soil organic matter by mechanisms other than nitrogen turnover.

If living roots exert a stimulative effect on denitrification, it could be assumed that this effect would occur in any soil containing living roots. To test this hypothesis, a comparison was made of nitrogen balances of permanent-grassland sods and of sown grass, after the application of labeled nitrate and ammonia (1960). Considerable losses of nitrogen occurred in both systems (Table 17, 18), a phenomenon confirming the above-mentioned hypothesis. In both grassland sods and sown grass a mechanism was shown to be operating during the ageing of the plants by which nitrogen was transported from the tops to the roots and in some cases also to the soil (Table 19). During this process the fertilizer nitrogen in the tops was partly replaced by unlabeled nitrogen (Table 18).

From the results presented in chapters III, IV and V it was concluded that living plants to a high degree influence the nitrogen cycle in permanent-grassland soils. An equation was derived which enabled the estimation of both mineralization and volatilization without making use of labeled nitrogen.

The stimulative effect of living plants on denitrification may be due, firstly to low oxygen tensions in the rhizosphere, caused by respiration of the root system and the microflora, and secondly to root excretions serving as a source of hydrogen donors during denitrification. By comparing oxygen consumption of permanent-grassland sods with living and killed roots, respectively, (the former was 28 times higher), it was shown that the effect of living plants on denitrification depends in the first place on the creation of low oxygen tensions which permit denitrification in the rhizosphere. The increased oxygen uptake in sods with living roots was found to depend for 66 per cent on the consumption by the roots, the remainder being consumed by the rhizosphere organisms. An adequate amount of hydrogen donors proved to be excreted by the roots of both perennial ryegrass and green peas (Fig. 3, 4), to enable a ready denitrification. Indications were obtained that the root excretions of perennial ryegrass were different from those of peas, the latter (possibly because of a larger proportion of amino acids) causing a higher denitrification rate. From the results obtained it was concluded that the rhizosphere constituted an excellent environment for the occurrence of the denitrification process (oxygen deficiency, sufficient amounts of hydrogen donors, large numbers of denitrifying organisms, and a continuous supply of nitrate after application of nitrate-containing fertilizers).

Since the operation of the nitrogen cycle in permanent-grassland soils was

shown to be largely the result of an interaction between microorganisms and higher plants, the microflora of these soils was studied under different climatological conditions. Actinomycetes generally represented more than 50 per cent of the total population (Table 21). The number of Gram-negative rods was found to be considerably higher and that of arthrobacters considerably lower than that of arable soil (Table 21), an observation which suggests that the bacterial population of permanent-grassland soils represents a rhizosphere flora. An investigation of the nutritional requirements of these bacteria revealed that about 80 per cent of the isolated strains were stimulated by amino acids (Table 22), an observation which underlined the rhizosphere character of the bacterial flora. Application of fertilizer nitrogen to grassland soils resulted in an increase of the number of Gram-negative rods (Table 23). It was supposed that the excretion of amino acids was stimulated as a consequence of fertilizer-nitrogen application.

A comparison of the denitrifying populations of grassland sods, with and without nitrate addition, revealed that under normal field conditions the population consisted mainly of *Bacillus*-species (*B. cereus, B. circulans, B. macerans, B. coagulans, B. laterosporus*), of which *B. cereus* was by far the most numerous. However, addition of nitrate resulted in the development of a flora of denitrifying bacteria which consisted mainly of *Pseudomonas, Achromobacter* and *B. macerans*-strains (Table 25). It was concluded that the presence of denitrifying bacteria under normal field conditions is not the result of their denitrifying capacity, but depends on some other characteristic; furthermore, that no relationship exists between the original number of denitrifying organisms and the denitrifying capacity of the soil.

The capacity of denitrifying bacteria, isolated from permanent-grassland soil, to utilize root excretions as a source of hydrogen donors during denitrification, was investigated in a number of Warburg experiments in which pure cultures of these bacteria were added to sterile pea plants supplied with nitrate. The *Pseudomonas, Achromobacter* and *B. macerans*-strains, isolated during denitrification in this soil, produced gas from nitrate under these conditions. No gaseous reduction products were formed by *B. cereus, B. licheniformis* and some other bacteria (Fig. 5). The latter species, however, produced a considerable quantity of nitrite and also some ammonia (Table 26).

Since the results obtained suggested that particularly amino acids might have a stimulative effect on denitrification in soil, an investigation was made of the nutritional requirements of the isolated denitrifying bacteria. Amino acids were found to be indispensable for the growth of some of these organisms and to stimulate, either growth, or both growth and gas production of others (Table 27). It appeared that the denitrifying organisms could be divided into two groups. The bacteria of the *Pseudomonas*-group always required nitrate for growth under anaerobic conditions. Those of the *B. licheniformis*-group, however, were able to grow under anaerobic conditions in the absence of nitrate, when glucose was used as a carbon source, but they needed nitrate, when glycerol or lactate was substituted for glucose (Table 29). It was suggested that in the latter case nitrate was required to enable the first oxydation step of these compounds. Also in other respects large differences existed between both groups of organisms (slow denitrification and low production of cell material during denitrification by the bacteria of the *B. licheniformis*-group as compared with those of the *Ps. aeruginosa*-group). The low yields

of cell material of the species of the *B. licheniformis*-group, when cultivated under anaerobic conditions in the presence of nitrate, indicate that these species are at a disadvantage in the competition with organisms of the *Ps. aeruginosa*-group during denitrification in the soil.

To obtain more information concerning the observed differences between both groups of denitrifying organisms, the effect of various hydrogen donors on denitrification by *Ps. aeruginosa* and *B. licheniformis* was investigated. Washed cells of these bacteria, incubated anaerobically in Warburg vessels, were supplied with glucose, glutamic acid, or a mixture of both compounds. In the case of *Ps. aeruginosa*, a higher denitrification rate was obtained with glutamic acid than with glucose, while the mixture of both compounds gave the highest value (Fig. 6). Substitution of nitrite for nitrate as a hydrogen acceptor, raised the denitrification rate considerably (Fig. 8). It was concluded that the denitrification rate was not limited by the nitratase-activity, but by the activity of the dehydrogenases involved in the dissimilation of glucose and glutamic acid. The favourable effect of glutamic acid was found to be due to the relatively high activity of glutamic-acid dehydrogenase in connection with denitrification, resulting in the accumulation of α-ketoglutaric acid. Glutamic-acid dehydrogenase was present during denitrification as a constitutive enzyme (Fig. 11), whereas the enzymes responsible for the dissimilation of glucose had to be formed adaptively (Fig. 10).

Different results were obtained with *B. licheniformis*. No gaseous reduction products were formed from nitrate by washed cells of this species under anaerobic conditions in the presence of various hydrogen donors (Table 31). However, in the presence of glucose, a considerable nitrite production was observed, which was doubled when in addition glutamic acid was added (Table 32). No nitrite was formed when glutamic acid was added separately. These results could be explained by the fact that uptake of glutamic acid under anaerobic conditions is coupled with glucose consumption (Table 33). Amino acids were found to be required for the growth of *B. licheniformis* under anaerobic conditions, an observation which indicates that the nutritional requirements of this species are more complex than those of *Ps. aeruginosa*. The consumption of glucose by *B. licheniformis* under anaerobic conditions in the presence of nitrate, proceeded independently of the dissimilatory nitrate reduction (Table 35); however, more carbon dioxide was produced in the presence of nitrate than in its absence, indicating that the presence of nitrate resulted in different products being formed from glucose. The nitrate-reductase activity of washed cells of *B. licheniformis* and of *B. cereus*, grown anaerobically in a medium containing glycerol as the carbon source and nitrate as the hydrogen acceptor, was found to be considerably higher than that of cells grown under similar conditions in a medium containing glucose (Table 37). It was suggested that the conditions under which these organisms possess an optimal nitrate-reductase activity will seldom be found in the soil. Evidence was obtained concerning the non-enzymatic nature of the formation of nitrous oxide in growing cultures of *B. licheniformis*.

Finally a study was made of the reduction of nitrate to ammonia during the denitrification process in the sods of permanent grassland, and of the contribution of *B. licheniformis* to this process. Labeled nitrate was added to small sods of permanent grassland which were incubated anaerobically. Although considerable quantities of ammonia were formed, as compared with the con-

trol sods (no nitrate addition), the ammonia originated mainly from deamination reactions and only to a small extent from nitrate (Table 39). When a pure culture of *B. licheniformis* was added to sterile root systems of pea plants supplied with labeled nitrate, only a small quantity of the ammonia formed was derived from added nitrate (Table 40.) It was concluded that the reduction of nitrate to ammonia during denitrification is of no importance in sods of permanent-grassland soils, and that the conditions under which this reduction is performed by *B. licheniformis* do not occur in the soil. The conditions under which *B. licheniformis* produces ammonia from nitrate were examined in some detail. The results of VERHOEVEN, who found a considerable ammonia production in shallow layers of a glucose-containing, mineral medium supplied with nitrate as the sole nitrogen source, were confirmed (Table 41). It appeared that washed cells grown strictly aerobically in such a medium, were able to reduce nitrate and nitrite dissimilatorily to ammonia without any adaptation period when submitted to anaerobic conditions (Table 42). Under the same experimental conditions nitrite but not nitrate was reduced to ammonia by washed cells of *B. subtilis*, a strictly aerobic organism. When *B. licheniformis* was cultivated aerobically in a nutrient medium with ammonium nitrate or ammonium sulphate as the sole nitrogen source, the dissimilatory formation of ammonia from nitrate by washed cells was completely inhibited (Fig. 13). These results suggest a close relationship between the assimilatory and dissimilatory nitrate-reducing enzyme systems of *B. licheniformis*. Some aspects of nitrate reduction, *viz.* common features of assimilatory and dissimilatory nitrate reduction, and the coupling of energy-yielding reactions to dissimilatory nitrate reduction, were briefly discussed.

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SAMENVATTING

Het feit dat op blijvend grasland na een stikstofbemesting zelden meer dan 60 procent van de toegediende stikstof in de bovengrondse delen van het gras wordt aangetroffen, vormde het uitgangspunt van de hier beschreven onderzoekingen. Getracht werd, na te gaan, waar het resterende deel van de stikstofbemesting blijft. Vastlegging in wortels, micro-organismen en in organische stof van de grond, uitspoeling en vervluchtiging kwamen als mogelijke bronnen van verlies het meest in aanmerking. De grote hoeveelheid stikstof, aanwezig in de zode van blijvend grasland, maakte het onmogelijk door middel van conventionele bepalingsmethoden het niet-teruggevonden deel van de

bemestingsstikstof op te sporen. De meeste experimenten werden daarom uitgevoerd met gemerkte stikstof onder omstandigheden, welke die van de praktijk zo dicht mogelijk benaderden.

In oriënterende proeven (1958) werd gemerkte nitraatstikstof toegediend aan zoden van blijvend grasland, die afkomstig waren van veen-, klei- en zandgrond. De zoden werden geplaatst in potten, die van onderen waren gesloten om uitspoeling te voorkomen, en gebracht op 80 of 95 procent van de watercapaciteit. Na ongeveer 2 maanden werden stikstofbalansen van de zoden opgesteld door de bovengrondse delen (gras), wortels en grond te analyseren. De op deze wijze niet teruggevonden stikstof werd beschouwd als te zijn vervluchtigd. Op alle onderzochte grondsoorten bleek een aanzienlijk deel (15–37%) van de toegediende stikstof verloren te zijn gegaan, terwijl ook wortels en grond nog bemestingsstikstof bevatten (Tabel 6). Er bleek een lineaire, negatieve correlatie te bestaan tussen de hoeveelheid stikstof in het gras en de stikstofverliezen door vervluchtiging (Fig. 1). Alleen in de zoden afkomstig van veengrond traden bij het hoogste vochtgehalte grotere verliezen op dan bij het laagste vochtgehalte; op klei- en zandgrond was de invloed van het vochtgehalte niet significant.

In een tweede experiment met gemerkte stikstof werd de invloed van de snijfrequentie van het gras op de stikstofverdeling na een nitraatbemesting nagegaan. De snijfrequentie bleek op de vervluchtiging geen invloed te hebben; daarentegen bevatten wortels en grond meer bemestingsstikstof bij een geringere snijfrequentie (Tabel 7). Aanwijzingen werden verkregen, dat een deel van de door het gras opgenomen bemestingsstikstof in een later stadium naar wortels en grond getransporteerd was en gedeeltelijk door opgenomen bodemstikstof vervangen werd (Tabel 8).

In 1959 werden soortgelijke proeven gedaan met zoden van blijvend grasland op zandgrond (op 60% van de watercapaciteit), waaraan gemerkte nitraat- en ammoniakstikstof werden toegediend. Stikstofbalansen werden opgesteld op het moment, dat de minerale stikstof uit de grond verdwenen was (T_0) en een maand na deze datum (T_1). Verliezen van minerale stikstof traden zowel op na een nitraatgift (20 procent) als na een ammoniakgift (9 procent), zie Tabel 11. Tussen T_0 en T_1 namen de verliezen niet verder toe, waaruit bleek, dat geen stikstof door vervluchtiging uit het verouderende gras verloren was gegaan. In het geval van de nitraatbemesting werden de verliezen toegeschreven aan denitrificatie.

Om de invloed van levende planten op de stikstofkringloop (denitrificatie, mineralisatie en immobilisatie) te leren kennen, werden proeven uitgevoerd met graszoden waarvan de wortels gedood waren. Het bleek, dat in dergelijke zoden de mineralisatiesnelheid gering was, doch dat deze, door de zoden te homogeniseren, sterk verhoogd kon worden (Tabel 12). De nitrificatie in de graszoden was geremd in vergelijking met die in de ondergrond. Na toevoeging van gemerkte nitraat- en ammoniakstikstof aan zoden met gedode wortels, bleken de verliezen door vervluchtiging (9 procent na een nitraatgift en geen verliezen na een ammoniakgif) veel geringer te zijn dan in de proeven met levende planten (Tabel 14). Het verschil tussen beide systemen werd toegeschreven aan een stimulerende invloed van levende planten op de denitrificatie. Uit het experiment met gemerkte stikstof kon tevens worden afgeleid, dat, hoewel na een toediening van ammoniumsulfaat een aanzienlijke hoeveelheid bemestingsstikstof in de organische fractie van de grond werd vastgelegd, geen toe-

neming van de totale hoeveelheid bodemstikstof plaatsvond. Vastlegging in de organische fractie van de grond bleek uitsluitend het gevolg te zijn van uitwisseling van bemestingsstikstof tegen gemineraliseerde bodemstikstof als gevolg van microbiologische activiteit ("Turnover", vergl. ook pag. 8). Van het nitraat, toegediend aan zoden met dode wortels, werd slechts een gering percentage in de "Turnover" betrokken, hetgeen een voorkeur van bodem-micro-organismen voor ammoniakstikstof deed vermoeden. Door middel van proeven met reincultures van bodemorganismen kon deze hypothese worden bevestigd (Tabel 15). Voor de waarneming, dat na een nitraatbemesting van zoden met levende wortels, wèl een aanzienlijk deel van de bemestingsstikstof in de organische stof van de grond werd teruggevonden (Tabel 6, 11), werd geen verklaring gevonden.

Het waargenomen effect van levende planten op de denitrificatie deed veronderstellen dat deze invloed niet tot blijvend grasland beperkt zou zijn. Een vergelijking werd daarom gemaakt tussen stikstofbalansen van potten met ingezaaid gras en die van zoden van blijvend grasland, waaraan gemerkte nitraat- en ammoniakstikstof werden toegediend. Zowel in de potten met ingezaaid gras, als in die met graszoden, traden stikstofverliezen op (Tabel 17, 18), hetgeen in overeenstemming was met bovengemaakte veronderstelling. Bovendien bleek, dat tijdens het verouderen van het gras een aanzienlijk deel van de bemestingsstikstof naar de wortels en soms ook naar de grond werd getransporteerd (Tabel 19). Hierbij trad in het gras een gedeeltelijke vervanging op van bemestingsstikstof door gemineraliseerde bodemstikstof (Tabel 17, 18).

Uit de in de hoofdstukken III, IV en V vermelde resultaten werd de conclusie getrokken, dat levende planten op blijvend grasland de stikstofkringloop in de grond in sterke mate beïnvloeden, zowel door een verhoging van de denitrificatie, als door een voortdurende opname en afgifte van stikstofverbindingen. Aan de hand van het beschikbare cijfermateriaal werd een methode ontworpen, die het mogelijk maakte, zowel mineralisatie als denitrificatie met behulp van ongemerkte stikstof te bepalen.

Verondersteld werd, dat het stimulerende effect van levende planten op de denitrificatie wordt veroorzaakt door de zuurstofconsumptie van wortels en in de rhizosfeer voorkomende micro-organismen, en door uitscheiding van stoffen, die tijdens het denitrificatieproces als substraat en waterstofdonors van de denitrificerende bacteriën fungeren. Door de zuufstofopname van zoden met levende wortels te vergelijken met die van zoden met dode wortels (de eerste was 28 maal zo hoog), kon worden aangetoond, dat het effect van levende planten op de denitrificatie in de eerste plaats dient te worden toegeschreven aan een verhoogde zuurstofconsumptie in de rhizosfeer. Tweederde van deze verhoogde zuurstofopname bleek voor rekening van de wortels te komen, het resterende deel voor die van de rhizosfeerorganismen. Daarnaast bleken zowel wortels van Engels raaigras als die van erwten voldoende waterstofdonors uit te scheiden om een aanzienlijke denitrificatie mogelijk te maken (Fig. 3, 4). De door erwten uitgescheiden stoffen stimuleerden de denitrificatie sterker dan die van Engels raaigras. Vermoed werd, dat dit berustte op een relatief grotere uitscheiding van aminozuren door wortels van erwten. Uit de verkregen resultaten werd geconcludeerd, dat de rhizosfeer een ideaal milieu is voor het optreden van denitrificatie (zuurstoftekort, voldoende waterstofdonors, grote aantallen denitrificerende organismen en, na

een bemesting met nitraat, een voortdurende aanvoer van deze verbinding).

Een onderzoek van de micro-organismen, aanwezig in de zode van blijvend grasland onder verschillende klimatologische omstandigheden, leerde, dat actinomyceten in het algemeen meer dan 50 procent van de totale populatie uitmaakten (Tabel 21). De verdeling van de bacteriën over Gram-negatieve staafjes, arthrobacters en bacillen deed vermoeden, dat de bacteriepopulatie op blijvend grasland als een rhizosfeerflora beschouwd kan worden. Een onderzoek naar de voedingsbehoeften van deze bacterieflora bevestigde deze veronderstelling: een hoog percentage van de bacteriën had aminozuren nodig voor hun groei of werd sterk door deze verbindingen gestimuleerd (Tabel 22). Onder invloed van een stikstofbemesting nam het aantal micro-organismen in grasland sterk toe (Tabel 23). Deze toename was bijna uitsluitend het gevolg van een stijging van het aantal Gram-negatieve staafjes (Tabel 24). De resultaten gaven aanleiding te veronderstellen, dat onder invloed van een stikstofbemesting speciaal de uitscheiding van aminozuren sterk wordt bevorderd.

Van de organismen met denitrificerend vermogen werden in de zode van blijvend grasland bijna uitsluitend *Bacillus*-soorten aangetroffen. Van deze soorten waren *B. cereus* en *B. cereus* var. *mycoides* verreweg het talrijkst. Daarnaast werden *B. circulans, B. macerans, B. laterosporus* en *B. coagulans* geïsoleerd. Na incubatie onder anaërobe omstandigheden van graszoden, waaraan nitraat was toegevoegd, bleek de denitrificerende populatie bijna uitsluitend uit *Pseudomonas-, Achromobacter-* en *B. macerans*-stammen te bestaan, terwijl *B. cereus* en andere *Bacillus*-soorten niet in aantal waren toegenomen. Hieruit werd de conclusie getrokken, dat de niet-sporevormende bacteriën verantwoordelijk zijn voor de denitrificatie in graszoden, terwijl de aanwezigheid van de *Bacillus*-soorten onder normale omstandigheden niet te danken is aan hun denitrificerend vermogen, doch aan een andere eigenschap. Verder werd geconcludeerd dat het aantal denitrificerende organismen in een grond niets zegt over het denitrificerende vermogen van die grond.

In een aantal Warburg-experimenten, waarin reincultures van denitrificerende bacteriën samen met nitraat werden toegevoegd aan steriele erwteplanten, kon worden aangetoond, dat de tijdens het denitrificatieproces geïsoleerde *Pseudomonas-, Achromobacter-* en *B. macerans*-stammen in staat waren wortelexcreties als waterstofdonors voor de denitrificatie te gebruiken (Fig. 5). Daarentegen konden *B. cereus, B. licheniformis* en enkele andere organismen onder deze omstandigheden nitraat niet tot gasvormige producten reduceren. Wel bleek door *B. licheniformis* een aanzienlijke hoeveelheid nitriet uit nitraat gevormd te worden met daarnaast een geringe hoeveelheid ammoniak (Tabel 26).

Aangezien de verkregen resultaten deden vermoeden, dat speciaal aminozuren een stimulerend effect op het denitrificatieproces in de grond uitoefenen, werd een onderzoek ingesteld naar de voedingsbehoeften van de geïsoleerde denitrificerende organismen. Aminozuren bleken nodig te zijn voor de groei van sommige van deze organismen, terwijl van de resterende stammen, hetzij groei en gasproductie, hetzij alleen de groei gestimuleerd werd (Tabel 27). Het was mogelijk de denitrificerende bacteriën in twee groepen te verdelen. De organismen van de *Ps. aeruginosa*-groep hadden voor de groei onder anaërobe omstandigheden steeds nitraat nodig. De organismen van de *B. licheniformis*-groep daarentegen waren in staat onder anaërobe omstandigheden zonder nitraat te groeien, wanneer glucose als koolstofbron werd gebruikt. Met

glycerol of melkzuur als koolstofbron trad echter alleen bij aanwezigheid van nitraat groei onder anaërobe omstandigheden op (Tabel 29). Verondersteld werd, dat nitraat in het laatste geval nodig was om de eerste oxydatietrap van glycerol en melkzuur mogelijk te maken. Ook in andere opzichten bleken grote verschillen tussen beide groepen denitrificerende organismen te bestaan. De droge-stof-productie tijdens de denitrificatie door soorten van de *Ps. aeruginosa*-groep was nauwelijks geringer dan die onder aërobe omstandigheden. Nitraat was binnen enkele dagen uit het medium verdwenen. Bij de organismen van de *B. licheniformis*-groep was de droge-stof-productie tijdens de denitrificatie nooit hoger dan die van de meeste gistingen (vaak lager), terwijl nitraat meestal na 14 dagen nog in het medium was aan te tonen. De geringe droge-stof-productie door de organismen van de *B. licheniformis*-groep doet verwachten, dat deze organismen tijdens het denitrificatieproces in de grond in de concurrentiestrijd met de organismen van de *Ps. aeruginosa* in het nadeel zijn.

Om meer gegevens te verkrijgen over de waargenomen verschillen tussen beide groepen van denitrificerende bacteriën, werd het effect van verschillende waterstofdonors op de denitrificatie door *Ps. aeruginosa* en *B. licheniformis* nagegaan. Aan gewassen-cel-suspensies van beide bacteriën, die onder anaërobe omstandigheden waren voorgekweekt op glutaminezuur en glucose, werden glucose, glutaminezuur of een combinatie van beide stoffen als waterstofdonor toegediend. De cellen werden na toevoeging van nitraat in Warburgvaten onder anaërobe omstandigheden geïncubeerd. Onder deze omstandigheden bracht glutaminezuur bij *Ps. aeruginosa* een hogere denitrificatiesnelheid teweeg dan glucose, terwijl de combinatie van beide stoffen een nog gunstiger effect had (Fig. 6). Wanneer nitriet als waterstofacceptor werd gebruikt inplaats van nitraat, werd een aanzienlijk hogere denitrificatiesnelheid geconstateerd (Fig. 8). Uit de verkregen resultaten kon worden afgeleid, dat niet de nitraat-reductase-activiteit doch de activiteit van de dehydrogenases, betrokken bij de dissimilatie van glucose en glutaminezuur, beperkend was voor de snelheid van het denitrificatieproces. Het gunstige effect van glutaminezuur werd veroorzaakt door een relatief snelle waterstofoverdracht tijdens de deaminering, waarbij α-ketoglutaarzuur opgehoopt werd. Glutaminezuurdehydrogenase was tijdens het denitrificatieproces als constitutief enzym aanwezig, terwijl de enzymen betrokken bij de afbraak van glucose, adaptief werden gevormd (Fig. 10, 11).

Met gewassen cellen van *B. licheniformis* werden geheel andere resultaten verkregen. Nitraat werd door deze cellen niet tot gasvormige producten gereduceerd (Tabel 31). Wel werd met glucose als waterstofdonor een aanzienlijke nitrietproductie gevonden, welke verdubbeld werd, als bovendien glutaminezuur werd toegevoegd (Tabel 32). Geen nitriet werd gevormd als glutaminezuur afzonderlijk werd toegevoegd. De verkregen resultaten bleken te berusten op het feit, dat voor de opname van glutaminezuur onder anaërobe omstandigheden de aanwezigheid van glucose in het medium noodzakelijk is (Tabel 33). Merkwaardigerwijs werd glutaminezuur onder aërobe omstandigheden wel afzonderlijk gedissimileerd (Fig. 12). Voor de groei van *B. licheniformis* onder anaërobe omstandigheden bleken aminozuren noodzakelijk te zijn, hetgeen wijst op hogere voedingseisen van deze bacterie dan die van *Ps. aeruginosa*. Voor de afbraak van glucose onder anaërobe omstandigheden door *B. licheniformis* was nitraat niet noodzakelijk (Tabel 35); wel werd bij

aanwezigheid van deze verbinding meer koolzuurgas geproduceerd dan zonder nitraat. Werden de cellen onder anaërobe omstandigheden voorgekweekt op glycerol inplaats van op glucose, dan nam de nitraat-reductase-activiteit sterk toe (Tabel 37). Hetzelfde werd bij *B. cereus* geconstateerd. Bovengenoemde resultaten gaven aanleiding te veronderstellen, dat de omstandigheden voor een optimale nitraat-reductase-activiteit in de grond voor deze soorten zelden vervuld zullen zijn. Wel zal dit het geval zijn in blikken met nitraat behandelde vleeswaren. In enkele oriënterende proeven met groeiende cultures van *B. licheniformis* werden tevens aanwijzingen verkregen, dat de reductie van nitraat tot N_2O een chemisch en niet een biologisch proces is.

Tenslotte werd nagegaan of in de zode van blijvend grasland tijdens het denitrificatieproces, nitraat tot ammoniak werd gereduceerd en of *B. licheniformis*, die onder bepaalde omstandigheden een reductie tot ammoniak uitvoert, bij dit proces betrokken was. Gemerkte nitraatstikstof werd toegevoegd aan kleine zoden van blijvend grasland, die vervolgens anaëroob geïncubeerd werden. Hoewel in vergelijking met het blanco object (zonder nitraat) een aanzienlijke ammoniakvorming optrad, bleek slechts 1 procent van het toegediende nitraat tot ammoniak te worden gereduceerd (Tabel 39). Het overgrote deel van de ammoniakfractie was door deaminering van organische stikstofverbindingen gevormd. In een experiment, waarin een reincultuur van *B. licheniformis* samen met gemerkte nitraatstikstof aan steriele erwteplanten werd toegevoegd, werd twee tot drie procent van de gegeven nitraat tot ammoniak gereduceerd (Tabel 40). Uit deze resultaten werd geconcludeerd, dat de reductie van nitraat tot ammoniak in zoden van blijvend grasland van geen betekenis is en dat de omstandigheden, waaronder *B. licheniformis* deze reductie uitvoert, in de grond niet voorkomen. Een poging werd ondernomen, deze omstandigheden nader te karakteriseren. De resultaten van VERHOEVEN, die sterke ammoniakvorming vond in een weinig geaëreerd, glucose bevattend, mineraal medium met nitraat als enige stikstofbron, werden bevestigd (Tabel 41). Het bleek, dat cellen, die strikt aëroob in een dergelijk medium waren gekweekt en na uitwassen anaëroob met nitraat werden geïncubeerd, deze verbinding zonder enige adaptatieperiode kwantitatief in ammoniak omzetten (Tabel 42). *B. subtilis*, een strikt aëroob organisme, reduceerde onder dezelfde omstandigheden nitriet wèl, doch nitraat niét tot ammoniak. Wanneer de cellen van *B. licheniformis* aëroob op ammoniumnitraat of ammoniumsulfaat werden gekweekt, volgde na uitwassen en incubatie onder anaërobe omstandigheden geen reductie tot ammoniak (Fig. 13). Uit deze resultaten bleek een nauwe samenhang tussen assimilatorische en dissimilatorische nitraatreductie door *B. licheniformis*. Enige aspecten van nitraatreductie, nl. de overeenkomst tussen assimilatorische en dissimilatorische nitraatreductie werden in het kort besproken.

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