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Denitrification, N_2-fixation and fermentation during anaerobic incubation of soils amended with glucose and nitrate

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Abstract Nitrate and glucose additions were investigated for their role in the C and N dynamics during anaerobic incubation of soil. A gas-flow soil core method was used, in which the net production of N_2, N_2O, NO, CO_2, and CH_4 under a He atmosphere could be monitored both accurately and frequently. In all experiments clayey silt loam soil samples were incubated for 9 days at 25 °C. Addition of nitrate (50 mg KNO_3-N kg^{-1} soil) had no effect on total denitrification and CO_2 production rates, while the N_2O/N_2 ratio was affected considerably. The cumulative N_2O production exceeded the cumulative N_2 production for 6 days in the treatment with nitrate addition, compared to 1.2 days in the unamended treatment. Glucose addition stimulated the microbial activity considerably. The denitrification rates were limited by the growth rate of the denitrifying population. During denitrification no significant differences were observed between the treatments with 700 mg glucose-C kg^{-1} and 4200 mg glucose-C kg^{-1}, both in combination with 50 mg KNO_3-N kg^{-1}. The N_2 production rates were remarkably low, until NO_3^- exhaustion caused rapid reduction of N_2O to N_2 at day 2. During the denitrification period 15–18 mg N kg^{-1} was immobilised in the growing biomass. After NO_3^- shortage, a second microbial population, capable of N_2-fixation, became increasingly important. This change was clearly reflected in the CO_2 production rates. Net volatile fatty acid (VFA) production was monitored during the net N_2-fixation period with acetate as the dominant product. N_2-fixation faded out, probably due to N_2 shortage, followed by increased VFA production. In the high C treatment butyrate became the most important VFA, while in the low C treatment acetate and butyrate were produced at equal rates. During denitrification no VFA accumulation occurred; this does not prove, however, that denitrification and fermentation appeared sequentially. The experiments illustrate clearly the interactions of C-availability, microbial population and nitrate availability as influencing factors on denitrification and fermentation.

Key words Denitrification · N_2-fixation · Fermentation · N_2O/N_2 ratio · C-availability

Introduction

During anaerobicity different biogeochemical processes can occur simultaneously or sequentially, e.g., denitrification, dissimilatory nitrate reduction (DNR), methanogenesis, fermentation, and N_2-fixation.

Denitrification causes a loss of nitrogen available to crops and is a potential environmental hazard, as N_2O is an important intermediate product of denitrification. N_2O is a greenhouse gas which is also involved in the catalytic destruction of the ozone layer (Crutzen 1981). Atmospheric N_2O concentrations increase and soils are estimated to contribute about 70% of the total anthropogenic N_2O emissions (Kroeze 1993). The amounts of N_2O produced during denitrification can range from 0 to the majority of denitrification products. The impact of several factors on the N_2O/N_2 ratio can largely be traced back to their influence on the relative availability of oxidant versus reductant (Hutchinson and Davidson 1993). Among the parameters explaining N_2O/N_2 ratios are the N-oxide concentration, organic C availability, O_2 availability, and ratios of enzyme activity. However, the interactions of these factors are not understood well enough to predict actual N_2O production rates for different conditions.

When studying denitrification using the acetylene (C_2H_2) inhibition technique (Yoshinari et al. 1977), other anaerobic processes, altering carbon and nitrogen availability in soils, are disturbed. For example, fermentation is inhibited by C_2H_2 (Flather and Beauchamp 1992). Under

Dedicated to Professor J.C.G. Ottow on the occasion of his 60th birthday

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undisturbed conditions fermentation would compete for easily available C-sources. On the other hand, fermentation products such as acetate, butyrate, and propionate are effective C-sources for denitrifiers (Paul et al. 1989). Fermentative bacteria can reduce NO_3^- to NH_4^+ (DNR), competing for available nitrate. Nitrogen-fixing *Clostridia* compete for available carbon as well. However, they are the fermentative organisms primarily affected by C_2H_2 (Flather and Beauchamp 1992). Furthermore, C_2H_2 can be used as a C-source by soil microorganisms during prolonged incubations (Yeomans and Beauchanp 1985).

In order to avoid all the above artefacts, we studied denitrification under a He atmosphere. The aim of the research was: (1) to study the influence of nitrate and glucose on both the amount and the dynamics of N_2O production through biological denitrification and (2) to monitor the influence of nitrate and glucose on other processes taking place during or after denitrification possibly competing for C and NO_3^-.

Materials and methods

Gas production was measured using a fully automated gas-flow soil core method. Our set-up consisted of nine Plexiglass tubes (diameter 6 cm, length 20 cm), each connected to an individual gas-tight circuit, to give nine replicates. Gas was continuously circulated through the circuits to avoid diffusional problems. The nine circuits were connected to a selection valve to enable automatic gas sampling. Gas samples were analysed simultaneously on two gas chromatographs, one with an electron capture detector and one with a thermal conductivity detector. Gases determined were N_2O, N_2, CO_2, O_2, NO, and CH_4. Gas production rates were calculated from the measured concentrations in the circulating gas and should be interpreted as net production rates. The net production rate maxima do not necessarily coincide with gross production rate maxima. A complete description of the set-up, analysis conditions, and accuracy of the method is given by Swerts et al. (1995).

The columns were packed with 460 g moist soil and incubated at 25 °C for 9 days using He as the circulation gas. Anaerobicity was obtained by flushing the soil cores for 10 min with He. Soil was sampled periodically for analysis of mineral N, water-soluble carbon (WSC), and volatile fatty acid (VFA) concentrations. Soil columns opened for soil sampling were afterwards excluded from the experiment, resulting in a reduction of the number of replicates from nine, at the beginning of the experiment, to five at the end.

Mineral N was analysed in a soil extract (25 g soil:50 ml KCl 1 *M*). Nitrate, NO_2^-, and NH_4^+ concentrations were determined colorimetrically on an Skalar autoanalyser. Water-soluble carbon was determined on a soil extract (35 g soil:100 ml H_2O_{dist}) using a persulphate oxidation method followed by gas chromatographic CO_2 analysis, according to McCardell and Fuhrmann (1992). Volatile fatty acids were analysed in a soil extract (30 g soil:30 ml cold H_2O_{dist}). After addition of 1 ml metaphosphoric acid (25%) to 3.5 ml extract, the samples were analysed on a gas chromatograph with a flame ionisation detector (column:chromosorb 101, oven:140 °C, det./inj. 240 °C, carrier N_2), using valeric acid as an internal standard. All extracts were prepared by shaking for 1 h on a rotary shaker, followed by 10 min centrifugation at 10000 rpm and filtration of the supernatant over a prefilter which was, for the VFA extract, followed by a 0.45-µm filter. Four treatments were applied as follows (Table 1):(1) 50 mg NO_3^--N kg^{-1} added as KNO_3 (N50), (2) 50 mg NO_3^--N kg^{-1} as KNO_3 and 700 mg C kg^{-1} as glucose (CN14), (3) 50 mg NO_3^--N kg^{-1} as KNO_3 and 4200 mg C kg^{-1} glucose (CN84), and (4) control 20 ml H_2O kg^{-1} (H_2O). The additions of treatments 1, 2, and 3 were dissolved in 18.2 ml H_2O kg^{-1}. After mixing, the soils were kept at 4 °C overnight, before starting the anaerobic incubations. Each treatment consisted of nine replicate soil columns.

To obtain additional data on NO_3^-, NH_4^+, WSC, and VFA concentrations for the CN84 treatment a different type of experiment was conducted (CN84bis). Sixty-two test tubes (diameter 2.5 cm, length 20 cm) were filled to 75% of their volume with 80 g soil to which 50 mg NO_3^--N kg^{-1} as KNO_3 and 4200 mg C kg^{-1} as glucose dissolved in 18.25 ml H_2O kg^{-1} had been added. The test tubes were stoppered with rubber caps, and the atmosphere was replaced by He by alternate evacuation with a vacuum pump and addition of He. This was done twice at the start of the experiment and once a day every 2nd day throughout the 9-day experiment. The soil was incubated at 25 °C in the dark. The number of tubes used for destructive sampling and the time intervals between sampling were varied according to the expected changes in mineral N, WSC, and VFA concentrations.

The soil used for all experiments was sampled from the upper 0–10 cm of a clayey silt loam soil (Mal, Belgium) with the following characteristics:texture:0–2 µm, 17%; 2–50 µm, 69%; >50 µm, 14%; pH_{H_2O}:6.7 (1:2.5 soil:water); pH_{KCl}:5.6 (1:2.5 soil:KCl 1 *M*); total organic carbon:12.3 mg C g^{-1} dry soil. The bulked samples were air dried to a moisture content of 180 mg water g^{-1} dry soil, sieved to pass a 2-mm sieve, mixed, and stored at 4 °C.

Results and discussion

The different treatments resulted, at the start of the anaerobic incubations, in the initial concentrations given in Table 1. The slight differences in initial NO_3^- concentrations can be attributed to low mineralization and nitrification rates during storage of the soil prior to use. The very low initial WSC concentrationn for treatment CN84 cannot be explained.

Addition of NO_3^- had little influence on total denitrification rates (Fig. 1). Initial denitrification rates of the H_2O treatment were even slightly higher than for the N50 treatment. These differences can be explained by a higher C-availability in the H_2O treatment, as reflected by the initial WSC concentrations and by the CO_2 production rates. The addition of NO_3^-, however, had a considerable influence on the N_2O/N_2 ratio of the produced denitrification gases,

Table 1 Overview of the nitrate and glucose additions and the resulting initial nitrate, ammonium, and WSC concentrations for the different treatments

Treatment	Addition		Initial concentration		
	NO_3^- (mg N kg^{-1})	Glucose (mg C kg^{-1})	NO_3^- (mg N kg^{-1}	NH_4^+ (mg N kg^{-1})	WSC (mg C kg^{-1})
H_2O	0	0	34.7±0.7	0.2±0.1	90.1±7.6
N50	50	0	88.4±2.0	0.0±0.0	49.6±2.6
CN14	50	700	99.7±3.6	0.0±0.0	954.6±6.5
CN84	50	4200	85.6±2.6	0.0±0.0	(596.9±66.9)
CN84bis	50	4200	86.7±1.6	0.3±0.1	4196.5±568.7

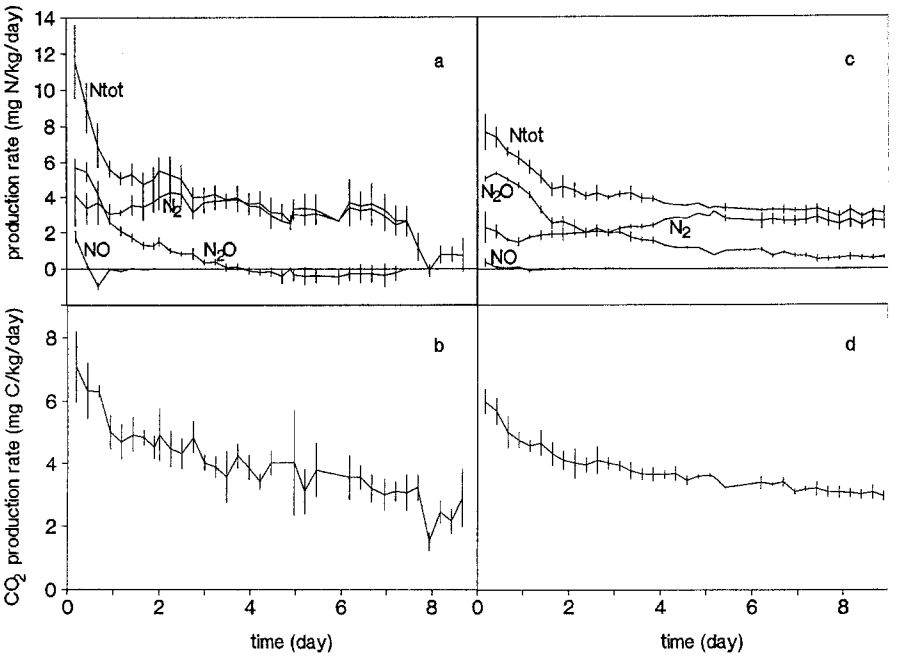

Fig. 1a–d Time course of the N_2, N_2O, NO, and N_{tot} (N_2+N_2O+NO) production rates (means±SD in mg N kg^{-1} day^{-1}) for the treatments H_2O (**a**) and N50 (**c**) and of CO_2 production rates (means±SD in mg C kg^{-1} day^{-1}) for the treatments H_2O (**b**) and N50 (**d**)

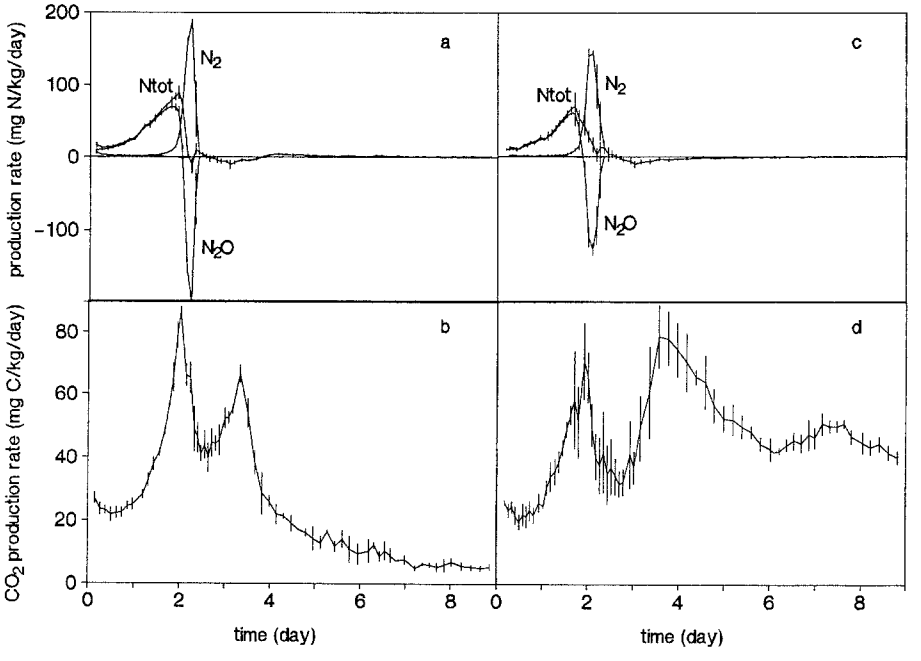

Fig. 2a–d Time course of the N_2, N_2O, and N_{tot} (N_2+N_2O+NO) production rates (means±SD in mg N kg^{-1} day^{-1}) for the treatments CN14 (**a**) and CN84 (**c**) and of CO_2 production rates (means±SD in mg C kg^{-1} day^{-1}) for the treatments CN14 (**b**) and CN84 (**d**)

although the effect of carbon availability cannot be entirely excluded. In the H_2O treatment N_2O production rates exceeded N_2 production rates for less than 1 day whereas N_2O was the major gas produced for 2.5 days in the N50 treatment. Cumulatively the N_2O production exceeded the N_2 production for 6 days in the N50 treatment compared to 1.2 days in the H_2O treatment (data not shown). N_2O production rates never reached zero during the N50 experiment, while N_2O production gradually changed into a net N_2O consumption at day 4 of the H_2O experiment. The drop in denitrification rates between days 7 and 8 in treatment H_2O was caused by NO_3^- exhaustion. The NO_3^- concentration at day 7.8 was 0 mg N kg^{-1}.

The 35 mg NO_3^--N kg^{-1} consumed in the H_2O treatment during the 9-day experiment was reasonably well balanced by a cumulative total denitrification of 32 mg N kg^{-1}. For treatment N50, 29 mg NO_3^--N kg^{-1} was consumed to produce 34 mg N kg^{-1} N gases over the 9-day period. The negligible low influence of NO_3^- on total denitrification clearly shows that carbon availability is a more important limiting factor for denitrification in the soil considered. This is consistent with our earlier work using the same soil (Swerts et al. 1996b), and with data of Lalisse-Grundmann et al. (1988) and Weier et al. (1993). Nitrate has, however, a clear influence on the partitioning of the denitrification products between N_2O and N_2. This corre-

sponds with the theory that when the availability of oxidant prevails over the supply of reductant, the substrate N oxide may be incompletely reduced, resulting in a larger N_2O/N_2 ratio of the end products (Hutchinson and Davidson 1993). CO_2 production rates were closely related to total denitrification rates. This is in accordance with data reviewed by Sahrawat and Keeney (1986), and Aulakh et al. (1992).

Glucose addition stimulated the microbial activity considerably as can be seen from the total denitrification rates and the CO_2 production rates for both treatment CN14 and treatment CN84 (Fig. 2). The exponential increase in rates reflects the growth of microorganisms on glucose. The initial N_2O production rate was ±8 mg N_2O-N kg^{-1} day^{-1}, increasing rapidly to reach 61.0 mg N_2O-N kg^{-1} day^{-1} after 1.6 days for treatment CN84, and 69.6 mg N_2O-N kg^{-1} day^{-1} after 1.8 days for treatment CN14. Exhaustion of nitrate caused the sharp drop in N_2O production rates. The first mineral N sampling was at day 2.9 for CN84 and day 2.1 for CN14. Nitrate and NH_4^+ concentrations were 0 mg NO_3^--N kg^{-1} and 0.2 mg NH_4^+-N kg^{-1} for CN84 and 2.3 mg NO_3^--N kg^{-1} and 0 mg NH_4^+-N kg^{-1} for CN14, indicating that at this stage of the experiment little to no net mineralisation or dissimilatory nitrate reduction (DNR) to ammonium had appeared. Experiment CN84bis provided more detailed data on mineral N evolution (data not shown). Nitrate concentrations decreased rapidly (power function) to reach 0 mg N kg^{-1} at day 1.99; at this time the NH_4^+ concentration was 0.44 mg N kg^{-1}. The NH_4^+ concentration increased subsequently to reach 1.30 mg N kg^{-1} at day 8.8.

During the first 2 days of the experiment N_2 production rates remained remarkably low. At NO_3^- shortage N_2O was rapidly reduced to N_2, causing a sharp increase in N_2 production rates. The CN84 treatment shows a maximum N_2 production rate of 143.8 mg N kg^{-1} day^{-1} at day 2.1, which coincides with the maximum net N_2O consumption of 127 mg N kg^{-1} day^{-1}. The maximum N_2 production rate of 187 mg N kg^{-1} day^{-1} for treatment CN14 was reached at day 2.2 as well as the maximum N_2O consumption rate of 199 mg N kg^{-1} day^{-1}.

The initial denitrification rates are in the same order of magnitude for all treatments (H_2O, N50, CN14, CN84). The denitrification rates decreased afterwards for the treatments H_2O and N50, while the rates for the CN14 and CN84 treatments increased. This is consistent with the findings of Smith and Tiedje (1979), who split up the denitrification process into phase I, lasting for 1–3 h in which the preexisting enzymatic capacity to denitrify is a more important limitation to denitrification than the supply of electron donor, and phase II in which enzyme synthesis and the potential for microbial growth increase the demand for electron donors, making carbon availability the more important limiting factor. As the soil used for all treatments had a similar history, the preexisting denitrifying enzyme levels were similar, resulting in an identical phase I. The indigenous population was comparable as well, resulting in the same growth response to glucose addition. In both treatments CN14 and CN84, growth of the denitrifier population was limiting denitrification rather than NO_3^- or available C. This explains the very similar production rates for N-gases and CO_2 until NO_3^- became limiting. For the CN14 treatment denitrification lasted slightly longer, resulting in higher N-gas and CO_2 production rates. This can be explained by the higher initial NO_3^- concentration of the CN14 treatment.

The high N_2O production rates are surprising under the given circumstances of high carbon availability, and even seem contradictory to the general theory that the N_2O/N_2 ratio decreases at high C availability. The high N_2O/N_2 ratios in this experiment seem to be due to the size of the denitrifying population rather than to the relative availability of oxidant versus reductant. As the population still needed to grow in response to the high C availability, both NO_3^- and available carbon were in excess. Because of the preferential acceptance of electrons by NO_3^- compared to N_2O, it was possible for N_2O to accumulate. The sudden high N_2O reduction rates at NO_3^- shortage indicate that N_2O reductase was not rate limiting, especially as these high rates occurred in a period of decreasing CO_2 production rates. N_2O was the major N-gas produced until day 2.0 for CN14 and day 1.8 for CN84.

The following cumulative maxima were reached: for CN14, total gaseous N 85 mg N kg^{-1} (day 2.6), N_2 59.5 mg N kg^{-1} (day 2.6), and N_2O 68.7 mg N kg^{-1} (day 2.0), reduced to 25.5 mg N_2O-N kg^{-1} (day 2.4); and for CN84, total gaseous N 67.2 mg N kg^{-1} (day 2.6), N_2 50 mg kg^{-1} (day 2.6), and N_2O 49.4 mg N kg^{-1} (day 1.8), reduced to 17.8 mg N_2O-N kg^{-1} (day 2.3). The nitrate not accounted for in denitrification products (18.4 mg N kg^{-1} for CN14; and 14.7 mg N kg^{-1} for CN84) was most likely immobilised in the growing biomass. Maximum cumulative denitrification losses were reached at day 2.6 for both treatments CN14 and CN84. Cumulative CO_2 production at that time was 100.4 mg CO_2-C kg^{-1} for CN14 and 92.4 mg CO_2-C kg^{-1} for CN84 (data not shown). Assuming that 60% of the decomposed organic C is incorporated into the microbial biomass and 40% is evolved as CO_2, 150.6 mg C kg^{-1} for CN14 and 138.6 mg C kg^{-1} for CN84 should be found incorporated in the active biomass. This would result in a C/N of the active biomass of 8.2 for CN14 and 9.4 for CN84, which is reasonably well within the range of reported C/N values found for newly synthesized biomass.

CO_2 production rates reached a first maximum [86.3 mg C kg^{-1} day^{-1} (day 2.0) for CN14; and 71.0 mg C kg^{-1} day^{-1} (day 1.9) for CN84] slightly after the maximum total denitrification rates (day 1.9 for CN14 and day 1.7 for CN84) and just before the maximal N_2 production rates (day 2.2 for CN14 and day 2.1 for CN84). As a response to nitrate exhaustion, at least part of the microbial population can be expected to die or to reduce its activity. CO_2 production rates reached a minimum [41.1 mg C kg^{-1} day^{-1} (day 2.5) for CN14, and 32 mg C kg^{-1} day^{-1} (day 2.7) for CN84] at the time of zero net denitrification.

At the time when NO_3^- concentrations became zero [day 2 for CN84 (from CN84bis), day 2.1 for CN14

(calculated from changes in N_2O and N_2 production rates and interpretation of the available data)], 0.81 mg CO_2-C had been produced for each milligram of NO_3^--N reduced for CN84. For CN14 this CO_2/NO_3^- ratio was 0.80. These values are lower than could be expected when only denitrification took place during the considered period. When glucose was the only C substrate and NO_3^- the only electron acceptor, reduction to N_2O would produce[1] 0.86 mg CO_2-C (mg NO_3^--N reduced)$^{-1}$ and reduction to N_2 would result[2] in a CO_2/NO_3^- ratio of 1.07. Dendooven et al. (1994) found a value of 1.49 for soils where only NO_3^- had been added, indicating CO_2 sources other than use of glucose by denitrifying microorganisms. The lower values found for CN14 and CN84 confirm the hypothesis that nitrate was lost in other ways than through denitrification (i.e. immobilisation).

Data on NO_3^- concentrations versus time were less detailed than data on gas production. Taking the total N gas production as a measure of NO_3^- reduction through denitrification (NO_2^- concentrations were 0 mg N kg^{-1} throughout the experiment), the ratio of CO_2-C produced for each milligram of NO_3^--N reduced could be studied in more detail (Fig. 3). The CO_2/NO_3^- ratios reached 2.26 (±0.50) for CN84 at day 0.41, and 1.82 (±0.08) for CN14 at day 0.26, and declined afterwards to reach a stable level of 0.81 (±0.03) for CN84 during the period day 1.36 to day 1.70, and of 0.67 (±0.02) for CN14 during day 1.20 to day 1.67, to increase again afterwards. The initial high ratios indicate CO_2 production from other sources than denitrification (not accompanied by N gas production). These could be microbial growth in combination with fermentation and/or DNR. The periods with constant ratios were preceding the maximum N_2O production rates and will probably be representative of systems where denitrifiers are the dominant population; however, the CO_2/NO_3^- ratios are too low to be entirely due to denitrification of NO_3^-, using glucose as a carbon source. Other carbon sources could provide lower CO_2/NO_3^- ratios. For example, the reduction of NO_3^- to N_2O using the VFA butyrate as a carbon source would produce[3] 0.69 mg CO_2-C (mg NO_3^--N reduced)$^{-1}$.

After the minimum in CO_2 production rates, another group of microorganisms, not dependent on denitrification, apparently became increasingly active and produced a second maximum in CO_2 production rates [66.5 mg C kg^{-1} day^{-1} (day 3.3) for CN14, and 79 mg C kg^{-1} day^{-1} (day 3.6) for CN84]. The rate of increase was comparable for treatments CN14 and CN84; however, the increase stopped earlier (day 3.3) for CN14, probably due to carbon limitations. At day 3.3, 141.4 mg CO_2-C kg^{-1} had been produced cumulatively in CN14. Taking into account the above-used C efficiency of 40% CO_2-C to 60% microbial C, 353.5 mg C would have been used at day 3.3, only about half of the added 700 mg glucose-C. However, in

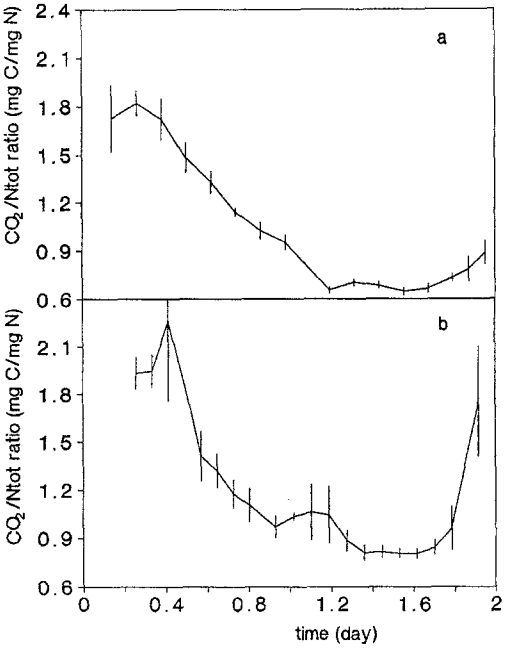

Fig. 3a,b Time course of the ratio of CO_2-C to N_{tot}-N (N_2+N_2O+NO) produced (means±SD in mg C mg^{-1} N), as a measure of the ratio of CO_2-C procuced for each mg NO_3^--N reduced through denitrification, for the treatments CN14 (**a**) and CN84 (**b**). Data are shown until day 2. After this time N_{tot} approached zero and became negative, causing extreme CO_2/N_{tot} values

aerobically incubated soils with high glucose additions a rapid uptake of glucose has been described (Bremner and Van Kessel 1991; Zagal and Persson 1994), not necessarily accompanied by biomass formation. Glucose or intermediate products can accumulate (intracellular reserve materials, extracellular polymers, and a variety of low molecular weight metabolites) without being used in cell growth or cell maintenance, resulting in biomass C/N ratios of up to 26 and in high apparent C use efficiencies. As such glucose availability could have limited the biomass activity at day 3.3 for CN14. Over the same period the WSC concentration of the CN84bis treatment had decreased by 1000 mg C kg^{-1}. The initial WSC concentration of CN14 was 954 mg C kg^{-1}. For the CN14 and CN84 treatments no detailed data on WSC versus time are available.

The second peak in CO_2 production rates was accompanied by net N_2-fixation. N_2-fixation cannot be measured directly in denitrification studies using the C_2H_2 inhibition method. Maximum N_2 fixation rates (10.5 mg N kg^{-1} day^{-1} for CN14 and 10.6 mg N kg^{-1} day^{-1} for CN84) were reached at day 3.0. At that time N_2 concentrations in the recirculating gas had dropped to 1.86% (CN14) and 1.66% (CN84).

For experiment CN84bis VFA concentrations remained at 0 mg C kg^{-1} until day 2 (Fig. 4). At day 2, the time of the first peak in CO_2 production rates, low acetate concentrations were measured, and butyrate appeared from day 2.85 on. Acetate concentrations exceeded butyrate concentrations until day 3.75; from then on VFA concentrations increased rapidly. At day 8.75 butyrate concentrations were

[1] $2(CH_2O)+2NO_3^-+2H^+ \rightarrow 2CO_2+N_2O+3H_2O$
[2] $5(CH_2O)+4NO_3^-+4H^+ \rightarrow 5CO_2+2N_2+7H_2O$
[3] $2C_4H_8O_2+10NO_3^-+10H^+ \rightarrow 8CO_2+5N_2O+13H_2O$

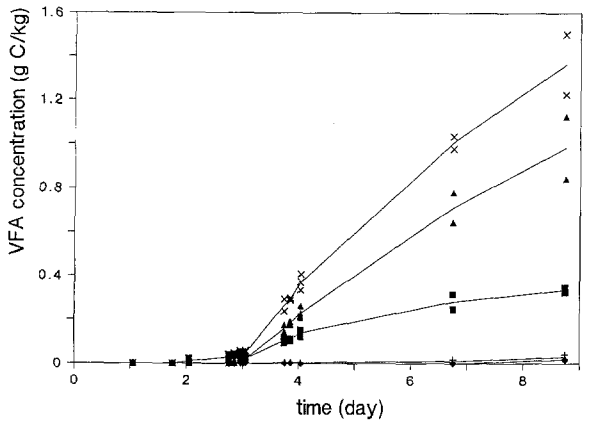

Fig. 4 Time course of volatile fatty acid concentrations [measured values (*symbols*) and means (*lines*) in g C kg^{-1}] for treatment CN84bis ▲ butyrate, ■ acetate, + propionate, ◆ isobutyrate, × sum of all VFAs

much higher than acetate concentrations (983 mg butyrate-C kg^{-1}, and 335 mg acetate-C kg^{-1}), and total VFV concentrations reached 1365 mg C kg^{-1}. Propionate and isobutyrate were minor fermentation products, reaching 30 mg propionate-C kg^{-1}, and 17 mg isobutyrate-C kg^{-1} at day 8.75. For experiments CN14 and CN84 less detail on VFA concentrations is available. For the CN14 treatment acetate and butyrate concentrations were about equal (±160 mg C kg^{-1}) and total VFA concentrations did not increase from day 7 to day 8.8 (±370 mg C kg^{-1}). For the CN84 treatment butyrate concentrations reached 430 mg C kg^{-1} at day 9.0, compared to 290 mg C kg^{-1} for acetate. Total VFA concentrations reached 780 mg C kg^{-1}. Data on VFA accumulation cannot be interpreted quantitatively, as part of the volatile substances will have been lost at sampling (CN14, CN84) or at evacuation of the test tubes (CN84bis).

During denitrification no VFA production was observed. Butyrate production became increasingly important as N_2-fixation faded out, due to N_2 shortage. Beauchamp et al. (1989) suggested that denitrification and fermentation occur simultaneously and argued that the VFA produced during fermentation serve as C sources for denitrifiers during anaerobic respiration. Paul et al. (1989) found that butyrate, propionate, and acetate were more efficient C-sources for denitrifiers than glucose and sucrose and attributed this to competition for available carbon between fermentative and denitrifying bacterial populations. From the obtained VFA data we cannot conclude whether denitrification and fermentation occurred simultaneously or sequentially. The above-mentioned high initial CO_2/NO_3^- ratios could be an indication of fermentation during the early denitrification phase, while the low CO_2/NO_3^- ratios during the period of high N_2O production rates could be due to VFA consumption by the growing denitrifier population. However, the VFA measured during and after N_2-fixation were not necessarily produced by fermentative populations active during denitrification.

Simultaneous N_2-fixation and denitrification could explain, in part, the high N_2O/N_2 ratios found during denitrification. Flather and Beauchamp (1992) found, however, that N_2-fixation (acetylene reduction method) was prevented until all nitrate had disappeared.

Non-symbiotic N_2-fixation under anaerobic circumstances is often attributed to Clostridia species (Flather and Beauchamp 1992; Rice and Paul 1971). Although fermentation to acetate produces more energy, butyrate is often formed to reduce the acid load and to avoid the accumulation of H_2 (Beauchamp et al. 1989). The observed acetate-butyrate molar ratios varied from 4.4 (day 2.9) to 0.6 (day 8.7) for CN84bis. This agrees with our earlier findings (Swerts et al. 1996a). For the CN14 treatment molar ratios varied from 3.7 (day 2.1) to 2.0 (day 8.9). The lower relative production of butyrate for CN14 can be explained by the reduced reducing power of this treatment with lower C availability. The maximum VFA concentrations reached in the CN14 treatment were comparable to those of the CN84bis treatment at day 3.75, the time at which C availability became limiting for the CN14 treatment. Flather and Beauchamp (1992) found in comparable experiments, after addition of 1000 mg glucose-C kg^{-1}, VFA concentrations and ratios in the same order of magnitude.

At day 9 WSC concentrations were 490 mg C kg^{-1}, 2272 mg C kg^{-1}, and 900 mg C kg^{-1} for CN14, CN84bis, and CN84. Volatile fatty acids are water extractable and contributed significantly to the WSC content (400 mg C kg^{-1}, 1400 mg C kg^{-1}, and 780 mg C kg^{-1} for CN14, CN84bis, and CN84). The WSC concentrations decreased for CN14 and CN84bis and remained fairly constant for CN84. This is in contrast with data presented by Swerts et al. (1996a), where WSC concentrations increased steadily under circumstances comparable to those for CN84 and CN84bis. The decrease in WSC concentrations can be explained by the rapid use of glucose by the microbial population, while, under anaerobic conditions with sufficient C available, the decay of complex organic molecules into water-soluble components is not likely to cause a considerable increase in the WSC content.

During the experiments no methane production was noticed.

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