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DENITRIFICATION RATE AND MICROBIAL DISTRIBUTION WITHIN HOMOGENEOUS MODEL SOIL AGGREGATES

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A b s t r a c t. Theoretical considerations of denitrification within soil aggregates lead to the conclusion that this process should increase with increasing aggregate size. The objective of this paper was to verify this statement for homogeneous, spherical soil aggregates. The artificial aggregates were formed from clay (48 % 2 μm) out of the Ah horizon of a Vertisol (Tröbersdorf) with the following diameters <2, 6.0, 10.0, 15.0, and 23.0 mm. Half of the aggregates were unamended and half NO_3^- (100 mg NO_3^--N kg^{-1})+C (400 mg of glucose C kg^{-1}) amended and then incubated at a constant soil water tension of 0.5 kPa for 4 days at 28 $\pm$1 °C in an atmosphere containing 0.03 l l^{-1} acetylene. The denitrification rate (as measured by N_2O evolution) and respiration rate (O_2 uptake) for all the aggregate sizes as well as the distribution of aerobic and anaerobic bacteria and denitrifiers in the biggest aggregates were studied. It was found that denitrification rate increased and respiration rate decreased with increasing aggregate size, both in amended and unamended soil aggregates. The number of denitrifiers and the total number of anaerobic bacteria increased while numbers of aerobic bacteria tended to decrease in the aggregate centre. The theoretical prediction of the dependence of denitrification rate on aggregate size was confirmed.

K e y w o r d s: denitrification, microbial distribution, soil aggregates

INTRODUCTION

It is generally believed that denitrification occurs in anoxic sites or pockets in the soil, which even exist in soil aggregates when the interaggregate pores contain almost atmospheric concentrations of oxygen [3]. Experimental confirmation of the existence of such anoxic zones within aggregates is provided by Greenwood and Goodman [4], and recently by Sextone *et al.* [10] and Stępniewski *et al.* [12]. This implies that denitrification should occur in larger soil aggregates at a certain moisture tension range close to saturation; the tension range being wider and denitrification rate being higher in larger aggregates. This concept has been used in several models of denitrification in aggregated soils [6,7,11].

However, the experimental results of Seech and Beauchamp [9] did not confirm this. These authors found that denitrification in natural aggregates isolated from the A horizon of a silt loam was highest in the smallest aggregates saturated with water, and that denitrification decreased with increasing diameter of the aggregates. The differences in the denitrification rates between aggregate sizes were partly reduced by replacement of air with He in the headspace, and partly by crushing of the aggregates size fractions. The authors found that denitrification

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rate in their soil was limited by carbon supply and suggested chemical and microbial differentiation of different size aggregates. In particular CO_2 production during aerobic incubation and biomass C both for aerobic and anaerobic incubations were greatest in the microaggregates and decreased with increasing aggregate size. In contrast Hattori [5] found that the microbial population in natural aggregates varied with the distance from the aggregate centre. In turn, Alef and Kleiner [1] found an increase in microbial activity, measured using the dimethylosulfoxide (DMSO) reduction method, in the centre of natural soil aggregates.

The objective of the present paper is to determine the denitrification rate of homogeneous spherical soil aggregates prepared in laboratory and differentiated in size, and to study distribution of aerobic, anaerobic and denitrifying bacteria within them. Supplementary measurements of aggregate respiration rate and oxygen distribution within the aggregates have also been taken.

MATERIAL AND METHODS

Soil material

The experiments were conducted using soil material from the Ah horizon of a Vertisol derived from Amaltheen clay (Tröbersdorf). It was characterized by 35 g kg^{-1} of organic carbon, 5 g kg^{-1} of total N, pH 6.5, 9 % of sand, 42 % silt and 49 % clay and a solid phase density of 2.49 Mg m^{-3}. The material was removed on Oct.1.1990, sieved through a 2 mm sieve two days later and then kept in a laboratory at a temp. 20 ±2 °C covered with plastic foil for 22 days. The soil water tension during the storage period was about 15 kPa.

Experiment I (with amended soil)

After 24 days from bringing it from the field the soil material was enriched with 100 mg kg^{-1} NO_3^--N and 400 mg kg^{-1} glucose-C by adding 1.082 g KNO_3 and 1.5 g glucose mixed with 10 g talc to 1.92 kg moist soil material with a water content of 0.28 kg kg^{-1} (water tension of 15 kPa). Aggregates 23, 15, 10, and 6 mm in diameter were produced in the polyvinyl chloride devices presented in Fig. 1. The soil material was used as a control. Although the aggregates swelled during saturation (see Table 1) the initial diameters will be used throughout the text.

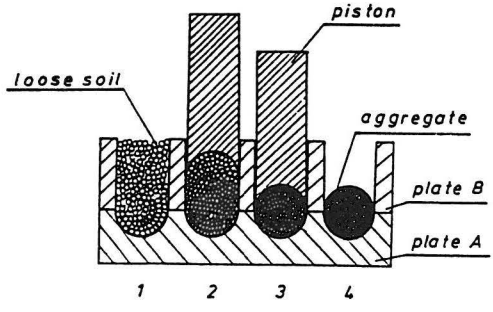

Fig. 1. Diagram of the device for aggregate production showing stages of the process (1-4). The presented device concerns15 mm aggregates (size 1:1). The devices contained 1, 10, 20, and 50 positions (holes) for the 23, 15, 10, and 6 mm aggregates, respectively. To prevent sticking of the aggregates to the piston they were covered with a thin polyethylene foil.

Table 1. Physical characteristic of the aggregates during incubation

Initial diameter (mm)	Average diameter at saturation* (mm)	Average volume at saturation* (cm^3)	Aggregate bulk density* (Mg m^{-3})	Total porosity E_o** (m^3 m^{-3})	Air-filled porosity E_9*** (g_o v/v)
23	25.8	8.935	0.941	63.0	2.1
15	17.3	2.705	0.962	62.1	n.d.
10	10.9	0.870	0.962	62.1	n.d.
6	6.7	0.157	0.959	62.0	n.d.
<2	n.d.	n.d.	n.d.	n.d.	n.d.

* - determined by weighing in paraffine, ** - calculated from bulk density and solid phase density, *** - determined by vacuum saturation in a dessicator at 5 hPa moisture tension plate, n.d. - not determined.

In order to assure access of acetylene to entire soil volume within the aggregates the aggregates were produced in a hermetically sealed plexiglass chamber (0.5 m^3 in volume) equipped with rubber gloves which enabled free manipulation inside. The soil material, the aggregate production devices and the incubation jars with moisture tension plates (Fig. 2) were placed in the chamber and the atmosphere was then enriched with 0.3 $l\,l^{-1}$ acetylene. The aggregates were produced in this atmosphere and then placed in the jars.

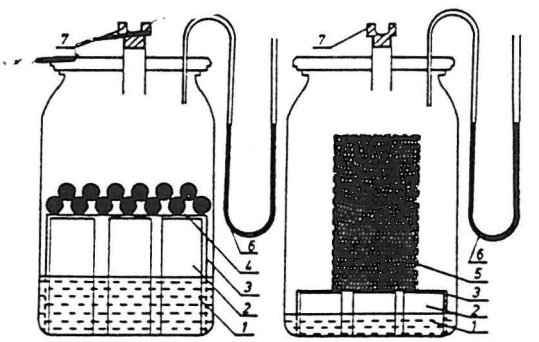

Fig. 2. Diagram of the incubation jars for the aggregates (left) and for the control (right). 1 - water, 2 - table supporting moisture tension plate, 3 - glass fiber tissue covering the table and immersed in water, 4 - soil aggregates, 5 - cylindrical nylon basket with the loose soil material <2 mm, 6 - mercury manometer, 7 - septum for gas sampling.

The jars were closed after all the aggregates have been made, i.e., 11 h after soil enrichment with KNO_3 and glucose and 8 h after placing the soil in the atmosphere containing acetylene. This amount of time was needed in order to complete all the operations. The time of contact with the tension plate at the moment of closing the jars was 6.5 h for 23 mm aggregates, 4.5 h for 15 mm aggregates, 3 h for 10 mm aggregates, 30 min for 6 mm aggregates and 1 min for <2 mm aggregates.

The physical configuration of the soil in the incubation jars is also shown in Fig. 2. Each jar contained 0.030 l of water which had been added before the jars were placed in the chamber. The aggregates lay on a glass fiber filter tissue immersed in water and the loose soil material was placed in a cylindrical nylon basket (mesh size of 1 mm). The mass of fresh soil material in each jar was 42 g (32.8 g of dry soil). The tension plates gave an average water tension -0.5 kPa with respect to the centre of the soil mass. The soil reached equilibrium within first 4 h.

The total volume of the jars was 0.28 l of which 0.013 l was occupied by solid phase of the soil, 0.039 l by water (in the soil and free), and 0.038 l by the 'table' supporting the soil. Thus, the total air volume was 0.19 l. Two jars were equipped with mercury manometers in order to follow the gas pressure changes.

The jars were incubated at a temperature of 28 ±1 °C for four days. Gas sampling was carried out after 12, 24, 48, and 96 h from the moment the jars were closed using evacuated vials (0.006 l in volume) through a septum in the jar lid. Sampling caused a reduction in the air pressure in the jar which was counteracted by leaving the needle in the septum open for 10 s in order to replace the gas sampled with the air from the atmosphere. The gas samples were analyzed for N_2O using a gas chromatography with an electron capture detector. The calculation of the total N denitrified was carried out by taking into account the solubility of N_2O in water assuming that the coefficient of N_2O solubility equals 0.63 $m^3\,m^{-3}$.

Experiment II (with unamended soil)

The second experiment was performed in the same way as the first one but original soil material without glucose and nitrate amendments was used. Other differences were as follows: soil material was stored for 9 days longer, i.e. 33 days after removing it from the field, and the gas sampling was not performed at the 12 h time point.

Oxygen concentration within the aggregates

Additional 23 mm aggregates were prepared for O_2 measurements (ϕ=-0.5 kPa).

Oxygen distribution within these aggregates was determined after 4, 8, 13, 30, and 50 h using the oxygen microelectrode described by Revsbech and Ward [8] and the technique described by Stępniewski *et al.* [12]. The electrode was driven continuously into the soil aggregates at a rate of 0.1 mm min^{-1}. The same technique was used to control oxygen distribution in the baskets at a level of 2 cm above the filtering tissue after 4 days of incubation.

Respiration measurements

After 96 h of incubation the oxygen content in the jars was measured with the same membrane oxygen microelectrode [9,12]. The electrode was installed in the flow through cell connected with 1 mm nylon tubes to syringe needle on the one side and to a water syphon on the other (Fig. 3). For respiration measurements the needle was introduced through a septum in the jar lid and the air in the jar was forced through the cell by pumping 0.020 l of water from the syringe into the incubation jar. Gas bubbles in the water syphon were an indication of the gas flow. The oxygen concentration was read after the value had stabilized. The minimum volume at which a stable value could be obtained was 0.002 l.

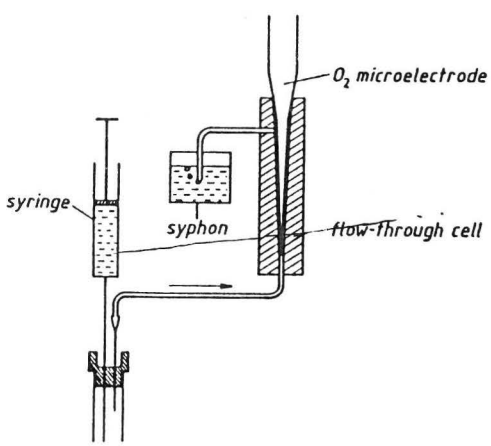

Fig. 3. Diagram of the system used for pushing air samples from the incubation jar with water filled syringe to oxygen microelectrode installed in a flow - through cell in order to determine soil respiration rate.

Separation of the aggregates into parts (peeling the aggregates)

After 96 h of incubation the 23 mm aggregates were taken out of the jars and frozen by immersing them in liquid nitrogen. After several minutes they were removed from it and exposed to an ambient temperature. Then, when the outer part of the aggregate had thawed it was pressed by hand (simultaneously turning it around) through a 20 mm wide hole in a stainless steel plate (1 mm thick). Thus the outer part corresponding to the distance from the aggregate centre >10 mm remained on the plate. The frozen central part of the aggregate was thawed further and then pressed through a 16 mm hole in the same way. Using this procedure the aggregate material was separated into three parts: outer (more then 10 mm from the centre), intermediate (8-10 mm from the centre) and inner part (<10 mm from the centre). Three aggregates of each of the 3 jars were peeled and the material of the aggregates from the same jar was mixed together. Thus, 3 samples of each aggregate part: outer, intermediate and inner, were obtained. A separate test showed that the procedure of freezing and thawing did not significantly influence the bacterial counts measured (Table 2).

From each of the three samples 2 g of soil (dry weight) were removed for immediate microbial analysis.

Table 2. Results of a test on the effect of freezing the soil in liquid nitrogen for 10 min and subsequent thawing on the microbial counts of the investigated soil after 20 months of storage at air-dry conditions

	Aerobes		Anaerobes		Denitrifiers	
	Frozen	Control	Frozen	Control	Frozen	Control
UCL	161000	161000	109000	152000	43.0	36.3
MPN	49000	49000	33000	46000	13.0	11.0
LCL	14800	14800	10000	14000	3.9	3.3

MPN - most probable number of bacteria in thousands per gram soil, UCL - upper 95 % confidence limit, LCL - lower 95 % confidence limit.

Microbiological assays

Microbiological assays comprised aerobic bacteria, anaerobic bacteria and denitrifier counts on the same medium (1 g KNO3 + 8 g of Difco nutrient broth in 1 l distilled water with pH 7) using the most probable number (MPN) technique under different conditions [2]. All microbial counts reported in this paper are expressed as number of organisms per gram of soil (dry-weight basis). Several minutes after thawing the aggregates, the soil samples were suspended in sterile water by shaking on a reciprocating shaker for 10 min and serially diluted (10-2 to 10-10) before being inoculated. All the media were incubated at 27 ±1 °C for 7 days with observations made daily.

Aerobic bacteria counts were determined during the incubation of test tubes with low head (10 cm) of the culture medium under aerobic conditions.

Anaerobic bacteria and denitrifiers were determined in test tubes with high (20 cm) head of the culture medium using Durham tubes. In order to create anaerobic conditions the incubation test tubes were closed with rubber stoppers and the remaining air (*ca* 0.001 l) was evacuated with a 0.020 l syringe. This resulted in a 10-20 fold reduction in the oxygen content in the test tube (in the liquid medium and in the gas space between the medium and the rubber stopper). The anaerobic bacteria counts were determined on the basis of turbidity observations. The number of the denitrifiers was calculated from the appearance of gas bubbles in the Durham tubes, and the disappearance of nitrate and the appearance of nitrite in the medium. The sampling of the medium for nitrate-nitrite tests was done with a sterile syringe through the rubber stopper. Occasionally, the gas from Durham tubes was analyzed using gas chromatography which confirmed the presence of high amounts of N_2O.

Statistical calculations

For the respiration data (Fig. 5) Tukey's 95 % confidence intervals for one way analysis of variance have been calculated. The same calculation procedure has been used for the denitrification data analysed for each sampling day separately (Figs 6 and 7).

The data concerning the most probable number of microorganisms (Table 2 and Figs 8 and 9) are presented together with their 95 % confidence limits calculated according to standard procedure of Cochran as described by Alexander [2]. The dilution ratio was 10, and the number of test tubes per dilution was 5 for the data in Table 2, and 3 - for the data in Figs 8 and 9.

It was not possible to treat the results from Figs 6 and 7 as two way classification (aggregate size x sampling day) because the assumption of equality of variances was not fulfilled for different sampling days.

RESULTS AND DISCUSSION

Oxygen distribution within unamended soil aggregates is shown in Fig. 4. As can be seen in this figure, anoxic zones appeared in the aggregates approximately one day after the aggregates had been produced and put on the 0.5 kPa tension plate. It is asumed that in the amended aggregates exhaustion of oxygen would proceed more vigorously. The measurement of oxygen distribution in the control soil material which was placed in the baskets after 4 days of incubation showed minimum values of oxygen partial pressure 17.3 kPa in the unamended soil and 16.1 kPa in the amended soil (at a level of 2 cm from the bottom of the basket). This indicates that the control soil material was essentially under aerobic conditions throughout the entire incubation period, whereas the biggest aggregates contained anoxic pockets from the end of the first incubation day.

The soil respiration rate which was measured by oxygen uptake is presented in Fig. 5. These values represent mean oxygen

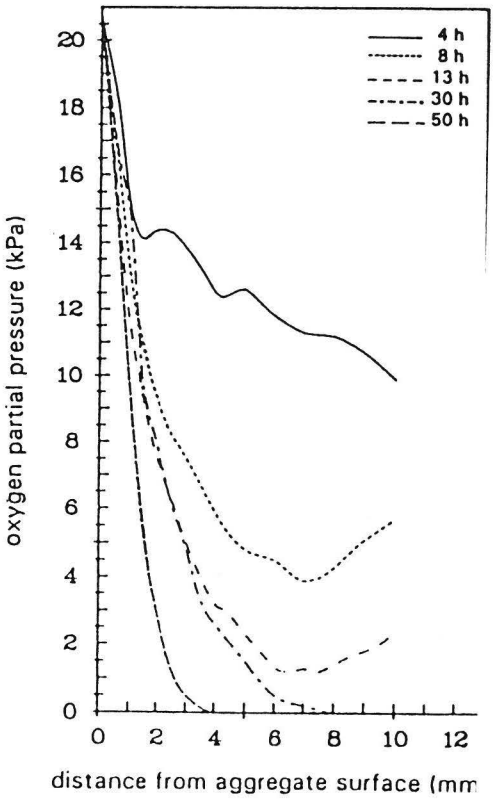

Fig. 4. Oxygen partial pressure distribution within unamended soil aggregates after 4, 8, 13, 30, and 50 h of contact with 5 hPa moisture tension plate (each curve is a mean of 2 aggregates).

uptake over the period of four days of incubation, although it is known that soil respiration rate changes with time, especially immediately after amendment with organic substrate. As can be seen the respiration rate was the highest for 6 mm aggregates (4 mg O_2 $kg^{-1}h^{-1}$) and decreased by 50 % for 15 and 23 mm aggregates. This corresponds to the mineralization of carbon over 4 days from 144 mg kg^{-1} to 72 mg kg^{-1} for the smallest and the biggest aggregates, respectively. This implies that in the 6 mm aggregates the soil volume under oxic conditions was approximately twice as much as compared to the biggest aggregates.

The denitrification rate is presented in Figs 6 and 7 for amended and unamended aggregates, respectively. The maximum amount of N denitrified during 4 days was 32 mg kg^{-1} in the amended soil and about 10 mg kg^{-1} in the unamended one. In both cases denitrification significantly increased with increase in aggregate diameter, in extreme cases (after 1 to 4 days) by a factor of 2.5 to 20 in the unamended soil and by a factor of 7 to 35 in the amended aggregates as compared to the control <2 mm fraction. It should be emphasized that in the amended soil aggregates the 10, 15, and 23 mm aggregates were characterized by

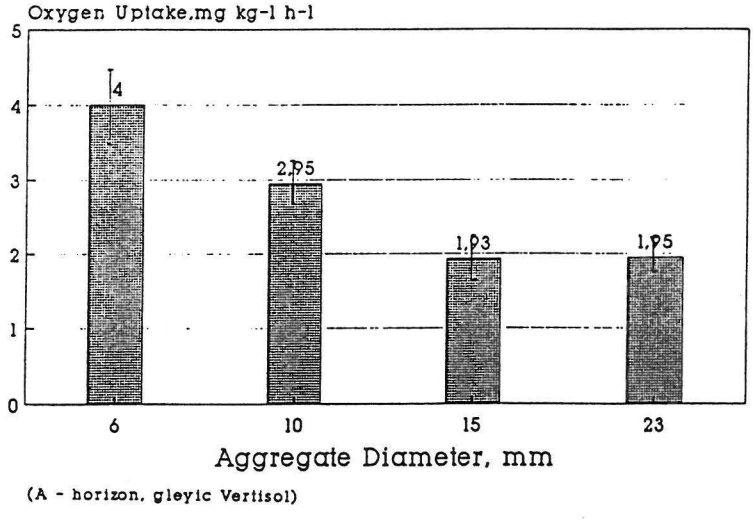

Fig. 5. Average respiration rates during 4 days of incubation for the amended aggregates as related to their diameter (the bars represent the lowest significant differences at P=0.05).

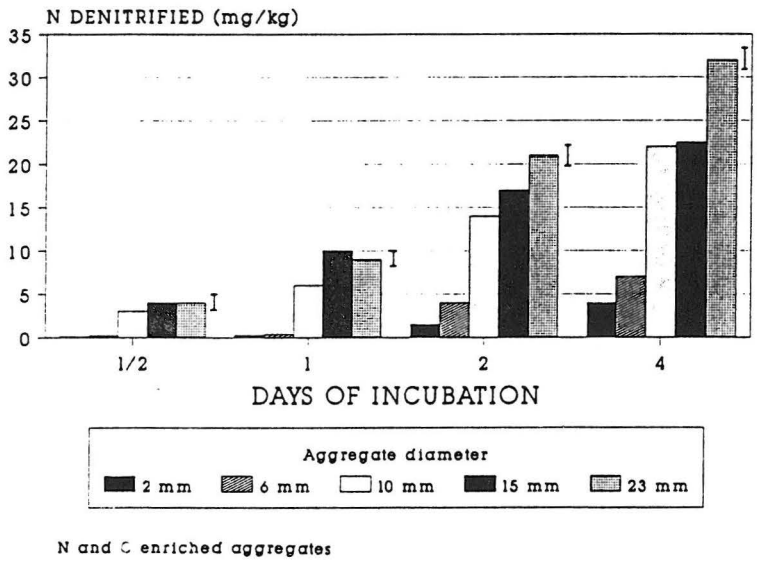

Fig. 6. The amount of N denitrified in different size aggregates amended with N+C after 1/2, 1, 2, and 4 days of incubation (the bars represent the lowest significant differences at P=0.05).

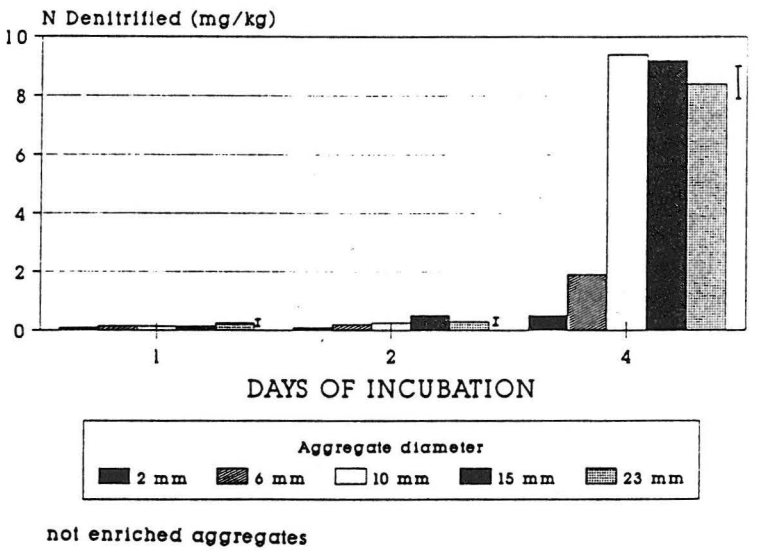

Fig. 7. The amount of N denitrified in different size unamended soil aggregates after 1, 2, and 4 days of incubation (the bars represent the lowest significant differences at P=0.05).

similar denitrification rates which were distinctly higher than those of the 6 mm and <2 mm aggregates. The results obtained here are in concert with the theoretical predictions [6,7,11] and in contrast to experimental results reported by Seech and Beauchamp [9] who found that in saturated, natural aggregates <0.25 mm the denitrification rate was higher then in bigger 5-10 mm and 10-20 mm aggregates.

The reasons of this discrepancy seem to be partly due to differences in the soil materials used and partly due to differences in physical conditions of incubation. The authors

mentioned above used natural soil aggregates while ours were artificial and due to this homogeneous, i.e., not differentiated spatially nor among the sizes. The biological activity of natural aggregates used by Seech and Beauchamp [9] (biomass C under oxic and anoxic incubation conditions, oxic CO_2 production at water tension -33 kPa, denitrification rate under an He headspace, as well as denitrification rate of crushed, saturated aggregates under an air headspace), was inversely related to the aggregate size. The spatial differentiation within the aggregates was not studied.

The second reason of the discrepancy between our and the quoted results can be connected with differences is spatial arrangement of the aggregates and soil particles during the incubation. It seems that water saturated <0.25 mm natural microaggregates as well as crushed material of bigger aggregates behaved in fact like one single 'macroaggregate' or clod. This is supported by the statement of the authors that 'the larger aggregates had a larger surface area exposed to O_2' during incubation under an air headspace and by the following observations:

- -increase of denitrification rate after crushing of the aggregates to the consistency of a powder to the level of that under an He headspace incubation;
- -disappearance of the inverse relationship of CO_2 production under an air headspace (at s.m. tension -33 kPa) on aggregates size after saturation.

This was avoided in our experiment by maintaining soil water tension at a level of -0.5 kPa, which kept empty the soil pores bigger than 0.6 mm. The problem of acetylene diffusion into the aggregate centre was solved by producing and incubation of the aggregates in an acetylene containing atmosphere. The limitation of denitrification by C substrate dominating in the quoted paper was not the case here as in our experiment the aggregate size limited denitrification rate in both experiments and soil enrichment with C+N only made the differences more distinct. It should be emphasized that a decisive difference exists between denitrification in fully saturated soil, i.e., when all the interaggregate pores are filled with water and the entire bulk of the soil behaves like a single block, and denitrification in quasi-saturated soil with the matrix potential of 0.5 to 2 kPa and more corresponding to air entry pressure into the interaggregate pores. In the second case the aggregate size and arrangement are of primary importance as the limit diffusion of oxygen and the denitrification process.

Thus our results seem to be applicable to situations where the aggregates and not the interaggregate pores are saturated with water and where the assumption of aggregate homogeneity is more or less valid. It seems that even when potential denitrification activity of microaggregates is higher then that of larger aggregates their role in denitrification should decrease and that of larger aggregates should increase with increasing soil water potential.

The results of microbial assays are shown in Figs 8 and 9. The total number of anaerobic bacteria was significantly higher (at $P=0.05$) in the aggregate center and decreased towards the surface. The number of denitrifiers in unamended soil showed significant increase by several orders of magnitude towards the aggregate center. In the amended treatment their number was significantly higher due to the enrichment but the tendency to increase towards the aggregate centre was not significant. For total aerobic bacteria the opposite is valid, i.e., their number showed a tendency to decrease towards the aggregate centre but this has not been proved to be significant. Generally a stimulation of the growth of anaerobes by anoxic conditions and of aerobes by oxic conditions was observed. We could not find this type of data in the literature and therefore are unable to make any comparisons. The effect of the enrichment with C+N should be discussed with a certain

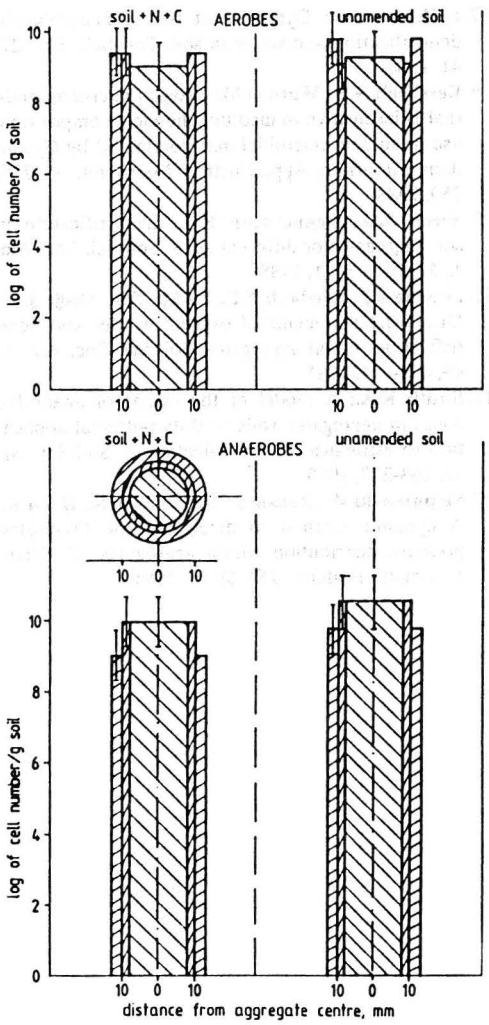

Fig. 8. Diagram of bacterial counts as related to the distance from centre of the biggest aggregates after 4 days of incubation at s.m. tension 5 hPa (the bars represent the lower an upper 95 % confidence limits).

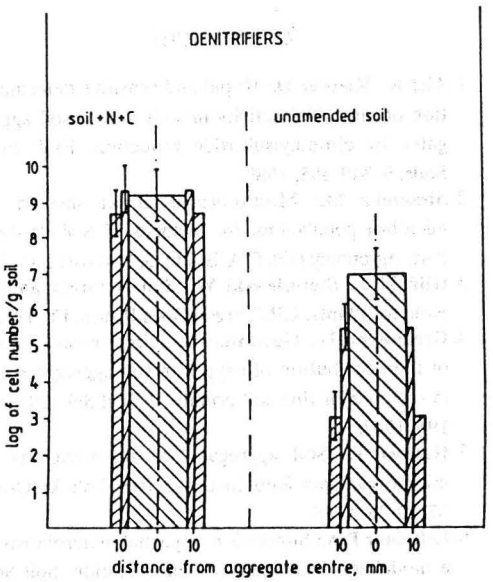

Fig. 9. Diagram of denitrifiers number as related to the distance from aggregates centre after 4 days of incubation at s.m. tension 5 hPa (the bars represent the lower and upper 95 % confidence limits).

amount of caution as the second experiment with unamended soil was performed 8 days after the first one, so the initial material may have changed slightly due to having being stored for 8 days longer. The observed insignificant tendency to obtain higher absolute values of both aerobic and anaerobic bacteria cells in unamended soil is striking and difficult to explain. Perhaps the problem of 'tiring' of the population, i.e., the decrease in its number after having reached the maximum due to substrate exhaustion was involved here. On the other hand the number of denitrifiers increased by several orders of magnitude due to N+C amendment. In the unamended soil they constituted not more then one part per thousand of all the anaerobes, whereas in the amended one their contribution reached 16 % of the total anaerobic bacteria.

CONCLUSIONS

It was found that in saturated homogeneous soil aggregates when the interaggregate spaces are air-filled the denitrification rate increased with increasing aggregate size indicating the physical particle arrangement as a limiting factor of the process. The existence of anaerobic zones within artificial aggregates was confirmed by direct measurements with a microelectrode. It has been also observed that initially homogeneous aggregates after four days of saturation become differentiated with respect to microbial population indicated by higher number of denitrifiers and of total anaerobes and by a slight tendency of lower number of aerobes in the aggregate centre.

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