Danish Research Service for Plant and Soil Science
Research Centre for Agriculture
State Laboratory for Soil and Crop Research
Soil Chemistry Department
DK-2800 Lyngby

Report no. 1803

Seasonal variation in numbers and activity of denitrifying bacteria in soil Taxonomy and physiological groups among isolates

Sæsonvariation i denitrificerende bakteriers antal og aktivitet i jord
Systematisk og fysiologisk karakterisering af isolater

Søren Christensen and G. J. Bonde

Summary
In a field experiment with addition of liquid manure (100 t per ha) or NH_4NO_3 (160 kg N per ha) the N-loss by denitrification was strongly influenced by changes in the soil environment. A change from moist soil containing 13–34 mg NO_3^--N per kg to dry soil containing 1–3 mg NO_3^--N per kg was followed by a 10–100 fold decrease in denitrification activity and a 10 fold decrease in MPN of denitrifying bacteria. NO_3^--reducing bacteria isolated from the MPN-tubes are divided into 3 physiological groups: NO_2^--formers with slight N_2O formation and N_2O- and N_2-formers respectively. Among the 3 groups N_2-formers were most prevalent irrespective of denitrification activity. The majority of isolates in all groups belonged to genus *Pseudomonas*.

Key words: Nitrogen loss, denitrification, *Pseudomonas*, slurry, fertilizer, N_2O, N_2.

Resumé
Kvælstoftabet ved denitrifikation var stærkt afhængigt af jordmiljøet efter tilførsel af svinegylle eller kalkammonsalpeter. Ved sammenligning af vandmættet jord indeholdende 13–34 ppm NO_3^--N med tør jord indeholdende 1–3 ppm NO_3^--N, fandtes en 10–100 gange højere denitrifikationsaktivitet og 10 gange flere denitrificerende bakterier i første tilfælde. Nitratreducerende bakterier, der producerer luftformigt kvælstof er opdelt i 3 fysiologiske grupper: NO_2^--dannere med svag N_2O produktion, N_2O-dannere og N_2-dannere. N_2-dannere var de hyppigst forekommende isolater ved både høj og lav denitrifikationsaktivitet. *Pseudomonas* var den mest udbredte bakterieslægt i alle grupper.

Nøgleord: Kvælstoftab, denitrifikation, *Pseudomonas*, gylle, handelsgødning, N_2O, N_2.

Introduction

In recent years new methodology has facilitated direct measurement of the nitrogen loss by denitrification. Gas chromatographic techniques (4) and labelling procedures with N-15 (6) and N-13 (11) have made it possible to measure denitrification in the field and in soil samples in the laboratory. Soil bacteria capable of denitrification and other N-gas forming reactions have been isolated and identified (5, 7). In a previous study of a soil kept fallow for more than 10 years, addition of liquid manure resulted in an increase in N-gas formation parallel with an increase in the number of isolates of fluorescent pseudomonads (1).

The aim of this study was to describe the influence of organic versus inorganic nitrogen amendments on the number and taxonomy of N-gas forming bacteria in the soil. A detailed description of the amount of N-gas formed is published in the preceeding paper (2).

Materials and methods

Study site

The investigation was made on a sandy loam soil at Roskilde Research Station (2). The field was cropped with spring barley both in the year of this experiment and the previous year. The soil samples were drawn from 2 treatments: 160 kg N per ha in limed NH_4NO_3 and 100 t liquid manure (pig slurry) containing 4.2% dry matter and 0.32% inorganic N, corresponding to 320 kg inorganic N per ha. Amendments of nitrogen were performed 11 April 1983.

Chemical analysis

Denitrification activity was measured in freshly collected soil samples from the 0–20 cm layer and placed in flasks. The accumulation of N_2O during the first 2–3 hours of incubation was measured on a gas chromatograph after the addition of 10% (v/v) acetylene (10). The concentration of N_2O in the headspace of septum-sealed MPN-tubes was determined on a gas chromatograph with a ^{63}Ni EC detector (1). The production of N_2O and N_2 by isolated soil bacteria was measured on gas chromatograph with ^{63}Ni EC-detector and thermal conductivity detector respectively (1). NO_3^- and NO_2^- in the growth medium of incubation flasks was determined by ion-selective electrode (Radiometer) and a diazotation reaction, respectively (1).

Microbiological analysis

Determination of the most probable number of soil bacteria followed the procedure previously described (1). Bacterial isolates were obtained on streak plates (nitrate agar) from the MPN-tubes positive for N_2-formation (bubble in minitube) or N_2O-formation (above 1% substrate-N in N_2O) (1). 200 ml nitrate broth (Difco) were added to flasks (500 ml) with 2 side arms and septum and autoclaved. To determine the products of nitrate reduction, the flasks were inoculated with the isolates, evacuated and refilled with helium 3 times. The flasks were incubated for 2 weeks at 10°C (1). Cultures reducing at least 0.1% of the substrate NO_3^--N into N_2O or N_2 were further identified to genus or species level (3).

Results

The N-loss was of similar size in the 2 treatments immediately before manure and fertilizer were

Table 1. Soil conditions at the samplings.

Date of sampling	Soil temp. °C	Soil water content	Soil nitrate content		N-loss by volatilization	
			160 kg N/ha	100 t slurry/ha	160 kg N/ha	100 t slurry/ha
		weight %	mg N/kg soil		μg N/kg soil/h	
11 April	6	20	10	10	3.8	3.7
9 May	10	18	34	22	1.5	6.5
4 August	20	5.5	2.9	1.4	0.12*	0.07*

* The figures are the mean of samplings 29 June and 27 September

Table 2. MPN counts ($\times 10^6$ per g soil) of NO_3^--reducing and N-gas producing bacteria.

Date of sampling	160 kg N/ha		100 t slurry/ha	
	NO_3^--red.[1]	N-gas	NO_3^--red.	N-gas
11 April[2]	14	9.1	24	15
9 May	24	20	86	42
4 August	3.8	2.9	5.8	3.6

[1] NO_3^--red.: NO_2^- in medium, above 1% of substrate-N in N_2O or bubble in Durham tube; N-gas: above 1% of substrate-N in N_2O or bubble in Durham tube. Figures are the result of 3 replicate determinations. See text for further evaluation.
[2] Soil sampled before amendment, the treatments should be considered as parallels. For further explanation see text.

applied. The soil contained 10 ppm NO_3^--N and had a moisture content corresponding to field capacity.

In May the soil was still very wet due to heavy precipitation in the preceeding period (Table 1). Both manuring and fertilization resulted in a 2–3 fold increase in the NO_3^--content. The gaseous N-loss was of comparable size in April and May. In August the soil had been dry for 1–2 months. The soil NO_3^--content was much decreased and the gaseous N-loss was an order of magnitude or more lower than in May.

In both treatments the MPN of nitrate reducers and N-gas producers showed a slight increase from April to May and then a sharp decrease from May to August (Table 2). The number of denitrifying bacteria accounted for about 2/3 of the bacteria reducing NO_3^- (Table 2). The large difference between replicates in MPN of both nitrate reducers and N-gas producers in April in the unamended soil illustrates the heterogenity of the site (Table 2).

Incubation experiments were done with 50–70 isolates at each of the 3 samplings. Among the N-gas forming isolates the NO_2^--formers typically transformed 80–90% of the NO_3^--N into NO_2^-- and 0.1–2% into N_2O (Table 3). A few isolates showed a more extended N-reduction. 11 out of 18 isolates belonged to genus *Pseudomonas* (Table 3). All but one of the N_2O-formers transformed 75–85% of the NO_3^--N into N_2O (Table 4). No NO_2^-- or N_2-accumulation was observed. All isolates were pseudomonads. The N_2-formers typically transformed 75–85% of the NO_3^--N into N_2 with 2 exceptions showing less extended NO_3^--reduction (Table 3). 30 out of 34 isolates were pseudomonads. The occurrence of N-gas forming isolates in % of the number of NO_3^--reducers is shown in Table 4. On the average, the NO_2^-, N_2O, and N_2-forming isolates comprised 17, 16 and 35% of the NO_3^--reducing isolates, respectively. There was no evident connection between prevalence of the different N-gas producers and season, nitrogen amendments or denitrification activity.

Discussion

In April and May the soil environment was favourable for denitrification as opposed to the conditions in August (Table 1). Correspondingly the N-loss was high at the 2 spring samplings compared to the summer sampling (Table 1).

Converting the N-loss to an area basis gives 0.1–0.5 kg N per ha per day in April and May compared to 0.01 kg N per ha per day in August in the 0–20 cm layer (soil bulk density = 1.5). The MPN of the denitrifying bacteria ranged from 9–42 $\times 10^6$ per g soil in April and May to 3 $\times 10^6$ per g in August. The decrease in the denitrifying and nitrate reducing bacteria from May to August in the slurry-treated soil was significant at the 90% level while the decrease in the NH_4NO_3-treated plot and the difference between treatments was not significant. In an earlier investigation with a soil kept fallow for more than 10 years, the MPN of denitrifying bacteria was 1–7 $\times 10^6$ per g soil under conditions where denitrification occurred while about 0.1–1 $\times 10^6$ per g soil at no denitrification (1). Similar results were obtained by others (8, 12) who found a change in MPN of denitrifying bacteria above one order of

magnitude when comparing seasons with different denitrification activity. On the other hand, comparing different cultivation practices giving a fourfold variation in denitrification activity, the MPN of denitrifying bacteria did not vary (9). This is in accordance with the results of the present study. It is suggested that the changes in numbers of denitrifying bacteria observed reflect changes in the total population rather than drastic changes in the fraction of the population able to denitrify.

Among the bacterial isolates producing N-gas, the N_2-formers were the most prevalent (table 4). It should be kept in mind that an enrichment procedure preceded the isolation. The occurrence frequency of a given physiological group among the isolated NO_3^--reducers will therefore be influenced by the competitive advantage of this group during enrichment compared to the other groups. The N-gas formation by pure cultures on NO_3^--broth gives no evidence of different competitive ability between N_2O- and N_2-formers during enrichment (1). The intrinsic rate of N-gas production by a NO_2^--former belonging to genus *Bacillus* was lower than that of N_2O- and N_2-formers (1). The competitive ability of a NO_2^--former is therefore regarded as low compared to the N_2O and N_2-formers. The results in Table 4 therefore indicate that the N_2-formers may be more important than the N_2O-formers for the denitrification in this soil, whereas the importance of the NO_2^--formers is difficult to rule out. The influence of different factors such as pH, NO_3^- and organic matter content on the different isolate types must be known before conclusions about their relative importance *in situ* are reached.

The fluorescent pseudomonads *(P. fluorescens, P. putida* and *P. aeruginosa)* accounted for one half and other pseudomonads for one third of the N-gas forming isolates (Table 3). This is in accordance with a previous study of a soil with low organic matter input (1). *Gamble et al.* (5) also found fluorescent pseudomonads as the most common denitrifying bacteria in a survey of world soils. A striking difference, however, was the high occurence of bacilli (1/4 of the N-gas forming isolates) in the above mentioned soil of low organic matter as compared to this study where only one isolate of genus *Bacillus* was found. This in agreement with a study of grass covered soil (13). Here unfertilized soil was dominated by bacilli while the population in fertilized soil shifted towards pseudomonads. The low number of NO_2^--forming bacilli isolated in this study compared to unfertilized soil should not be taken as conclusive about the occurrence of bacilli *in situ* as indicated above. The taxonomic and physiolgical groupings are not related: several examples are found in which bacterial isolates, placed in the same species group belong to 2 or all 3 physiological groups (Table 3).

It can be concluded that the physiolgical groups of N-gas formers are well separated as defined here. Apart from a single isolate, no transition forms are found between the N_2O-formers and the NO_2^--formers with less N_2O-formation. Likewise the N_2-formers with 2 exceptions converted the main part of the substrate N into N_2. No difference in the physiological or taxonomic composite of denitrifying bacteria was found comparing soil of different denitrification activity.

Table 4. Total number of NO_3^--reducing isolates cultivates at the 3 samplings and occurence of N-reducing ability in % of this number.

Date of sampling	Soil treatment	Total number of NO_3^--reducers	% of total number		
			$(NO_2^-+N_2O)$-formers	N_2O-formers	N_2-formers
11 April	[1]	26	15	27	27
9 May	NH_4NO_3	25	24	8	33
	slurry	28	15	10	50
4 August	NH_4NO_3	12	8	24	24
	slurry	8	24	12	39

[1] Soil sampled before nitrogen amendments.

Table 3. NO_3^--reduction by isolates of soil bacteria producing N-gas.

Date of sampling	Soil treatment	NO_2^--formers: isolate	NO_2^--formers: NO_3^--reduction ($NO_2^-/N_2O/N_2$)	N_2O-formers: isolate	N_2O-formers: NO_3^--reduction ($NO_2^-/N_2O/N_2$)	N_2-formers: isolate	N_2-formers: NO_3^--reduction ($NO_2^-/N_2O/N_2$)
	[1]	*Pseudomonas alcaligenes*	(98/0.7/0)	6× *Pseudomonas fluorescens*	(0/79/0)	*Pseudomonas fluorescens*	(0/0/93)
						Ps. putida	(0/0/91)
11 April		*Ps. putida*	(98/1/0)			*Ps. fluorescens*	(0/0/71)
	[1]	*Klebsiella sp.*	(91/1.0/0)			*Ps. putida*	(0/0/88)
		Bacillus mycoides	(53/11/0)			2× *Ps. aeruginosa*	(0/0/77)
						Cytophaga/flavobact.	(0/0/75)
		Ps. fluorescens	(98/0.1/0)	*Ps. putida*	(0/85/0)	*Ps. putida*	(0/0/77)
		2× *Ps. putida*	(77/2/0)	*Ps. diminuta*	(0/83/0)	*Ps. alcaligenes*	(0/0/71)
	NH_4NO_3	*Ps. pseudoalcaligenes*	(45/27/0)			2× *Ps. pseudoalcaligenes*	(0/0/76)
		Ps. pseudoalcaligenes	(97/1.3/0)			*Ps. vesciculare*	(0/0/58)
		Eschericia coli	(87/0.7/0)			*Ps. diminuta*	(0/0/77)
9 May						*Achromobacter xyloxidans*	(0/0/77)
						Cytophaga/flavobact.	(0.5/0/30)
		Ps. putida	(79/0.7/0)	*Ps. fluorescens*	(0/85/0)	*Ps. putida*	(0/0/75)
	Slurry	*Ps. cepacia*	(93/1.3/0)	*Ps. putida*	(0/77/0)	3× *Ps. alcaligenes*	(0/0/72)
		2× *Enterobacter aerogenes*	(82/0.3/0)	*Ps. vesciculare*	(0/85/0)	4× *Ps. pseudoalcaligenes*	(0/0/79)
						3× *Ps. cepacia*	(0/0/77)
						2× *Chromobact. violaceum*	(0/0/78)
						A. xyloxidans	(0/0/79)
	NH_4NO_3	*Ps. putida*	(87/7/0)	*Ps. fluorescens*	(0/75/0)	2× Ps. putida	(0/0/80)
		[2] (no. 34_1)	(45/0/25)	2× *Ps. putida*	(0/77/0)		
4 August	Slurry	*Ps. fluorescens*	(92/1.0/0)	*Ps. cepacia*	(0/1.0/0)	2× *Ps. putida*	(0/0/74)
		E. aerogenes	(85/2.3/0)			*Ps. diminuta*	(0/0/85)

[1] Soil sampled before the nitrogen amendments.
[2] Isolate was accidentially not identified.

Literature

1. *Christensen, S.* 1985a. Denitrification in an acid soil: Effects of slurry and potassium nitrate on the evolution of nitrous oxide and on nitrate-reducing bacteria. Soil Biol. and Biochem. 17. In press.
2. *Christensen, S.* 1985b. Denitrification in a sandy loam soil as influenced by climatic and soil conditions. Tidsskr. Planteavl 89, 351–365.
3. *Christensen, S. & Bonde, G. J.* 1985. N-gas production by isolates of soil bacteria as influenced by N-source and acetylene. Microbiol. Ecol. In press.
4. *Focht, D. D.* 1978. Methods for analysis of denitrification in soils. *In* Nitrogen in the environment Vol. 2, 433–490 (*D. R. Nielsen* and *J. G. MacDonald* eds), Academic Press.
5. *Gamble, T. N., Betlach, M. R. & Tiedje, J. M.* 1977. Numerically dominant denitrifying bacteria from world soils. Appl. and Environm. Microbiol. 33, 926–939.
6. *Rolston, D. E., Sharpley, A. N., Toy, D. W. & Broadbent, F. E.* 1982. Field measurement of denitrification III. Rates during irrigation cycle. Soil Sci. Soc. Am. J. 46, 289–296.
7. *Smith, M. S. & Zimmermann, K.* 1981. Nitrous oxide production by nondenitrifying soil nitrate reducers. Soil Sci. Soc. Am. J. 45, 865–871.
8. *Terry, R. E. & Tate, R. L.* 1980a. Effect of flooding on microbial activities in organic soils: Nitrogen transformations. Soil Sci. 129, 88–91.
9. *Terry, R. E. & Tate, R. L.* 1980b. Denitrification as a pathway for nitrate removal from organic soils. Soil Sci. 129, 162–166.
10. *Tiedje, J. M.* 1982. Denitrification. *In* Methods of Soil Analysis, Agronomy no. 9, part 2 (*A. L. Page, R. H. Miller* and *D. R. Keeney* eds), 1011–1026.
11. *Tiedje, J. M., Firestone, R. B., Firestone, M. K., Betlach, M. R., Smith, M. S. & Caskey, W. H.* 1979. Method for the production and use of nitrogen-13 in studies of denitrification. Soil Sci. Soc. Am. J. 43, 709–716.
12. *Valera, L. & Alexander, M.* 1961. Nutrition and physiology of denitrifying bacteria. Plant Soil 15, 268–280.
13. *Woldendorp, J. W.* 1963. The influence of living plants on denitrification. Medd. Landbouwhogeschool, Wageningen, 63, 1–100.

Manuscript received 10 June 1985.