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Chloramphenicol Inhibition of Denitrifying Enzyme Activity in Two Agricultural Soils

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Chloramphenicol, at concentrations greater than 0.1 g/liter (0.3 mM), inhibited the denitrifying enzyme activity (DEA) of slurries of humisol and sandy loam soils by disrupting the activity of existing nitrate reductase enzymes. When the concentration of chloramphenicol was increased from 0.1 to 2.0 g/liter (6.0 mM), the rate of nitrite production from nitrate decreased by 25 to 46%. The rate of NO production from nitrate decreased by 20 to 39%, and the rate of N_2O production from nitrate, in the presence of acetylene (DEA), decreased by 21 to 61%. The predicted values of DEA at 0 g of chloramphenicol/liter computed from linear regressions of DEA versus chloramphenicol concentration were 18 to 43% lower than DEA measurements made in the absence of chloramphenicol and within a few per cent of DEA rates measured in the presence of 0.1 g of chloramphenicol/liter. We conclude that DEA assays should be carried out with a single (0.1-g/liter) chloramphenicol concentration. Chloramphenicol at concentrations greater than 0.1 g/liter inhibits the activity of existing denitrifying enzymes and should not be used in DEA assays.

Measurements of denitrifying enzyme activity (DEA) were proposed by Smith and Tiedje (19) as a way of assessing the potential optimum activity of existing denitrifying enzymes in soil. DEA is determined by measuring the rate of N_2O production, in the presence of acetylene, from soil samples placed under anaerobic conditions and supplied with excess carbon source (glucose) and nitrate. The DEA assay has been widely used (7, 13, 15, 16, 18, 22) and is the recommended method for measuring potential DEA in soil (21).

Smith and Tiedje (19) suggested that chloramphenicol should be used in DEA assays to inhibit synthesis of new denitrifying enzymes while the activity of previously existing denitrifying enzymes was being measured. Recent work has indicated that the use of chloramphenicol to prevent the synthesis of new denitrifying enzymes during the DEA assay may have had previously unrecognized effects on denitrifying enzymes (3, 5, 17, 23). Several investigators have suggested that chloramphenicol may disrupt the activity of previously existing denitrifying enzymes in agricultural soils (17), aquifer sediments (3), and pure cultures of denitrifying bacteria (23).

Wu and Knowles (23) noted that chloramphenicol inhibited DEA at the level of nitrate reduction in pure cultures of both *Flexibacter canadensis* and "*Pseudomonas denitrificans*" and at the level of NO reduction in *F. canadensis*. We investigated the influence of chloramphenicol on the activity of the four denitrifying enzymes (24, 25) in two agricultural soils over a range of chloramphenicol concentrations commonly used in DEA assays.

MATERIALS AND METHODS

Soil. Humisol was collected from the Central Experimental Farm of Agriculture & Agri-Food Canada, Ottawa, Ontario, Canada, and is highly organic (58 to 62% weight loss on ignition [6]). St. Bernard sandy loam soil was collected from the Morgan Arboretum of McGill University, Ste.-Anne-de-Bellevue, Quebec, Canada. It is a mineral soil with a loss on ignition of 4.5% (6). Both soils were collected from the surface 10 cm and stored at 4°C. The soils were sieved through a 1.0-mm-mesh-size sieve prior to the start of experiments.

Net nitrite production from nitrate and nitrite reduction. Samples of between 20 (humisol) and 40 (sandy loam) g of soil were placed in 50 ml of assay solution containing phosphate buffer (50 mM; pH 7) supplemented with 10 mM nitrate, 10 mM glucose, and chloramphenicol (Sigma). Chloramphenicol was dissolved in the buffer solution, and the final concentration was varied from 0.1 to 2.0 g/liter. Incubations were carried out in 150-ml Erlenmeyer flasks capped with SubaSeals (William Freeman Co., Barnsley, England). Anaerobic conditions were established in the flasks by evacuating and flushing them three times with N_2. The flasks were vented to atmospheric pressure prior to the beginning of experiments and placed on a rotary shaker at 200 rpm. All treatments were in triplicate. The flasks containing humisol were sampled each hour for 5 h. The flasks containing sandy loam soil were sampled every 2 h for a total of 10 h. The time course of nitrite production was linear over these periods.

Nitrite consumption was measured by monitoring the consumption of nitrite that was produced by reduction of nitrate added in the assay solution. Nitrite was not added directly to soil slurries because nitrite added directly to soil can undergo chemical decomposition to NO (4). Flasks containing soil and assay solution as described above were evacuated and flushed with N_2 and placed on a rotary shaker overnight. Nitrite concentrations in the humisol and sandy loam reached a maximum after 28 and 30 h, respectively, at which time an ethanolic solution of chloramphenicol (between 1 and 2% [vol/vol], final concentration) was injected through the stoppers into the flasks. Ethanol at these concentrations did not affect the rate of nitrite consumption by either soil. Consumption of the indigenously produced nitrite was measured at 0.5-h intervals, over a period of 3 h for the humisol and at hourly intervals over a period of 6 h for the sandy loam. Time courses for nitrite consumption were linear over the sampling period in both soils.

Net production of NO from nitrate and NO reduction. NO production was measured in flasks containing 10 g of soil and 25 ml of assay solution (phosphate buffer, 50 mM; pH 7) supplemented with 10 mM nitrate, 10 mM glucose, and chloramphenicol. The flasks were evacuated, flushed with N_2, vented to atmospheric pressure and shaken at 200 rpm. The headspaces were sampled for NO analysis at 20-min intervals over a period of 100 min, and NO production was linear over this time interval.

NO consumption was monitored in incubations in which NO produced from endogenous soil nitrate was depleted. Flasks containing 10 g of soil and assay solution which was not supplemented with nitrate or chloramphenicol were evacuated, flushed with N_2, and shaken overnight. The next morning, NO produced from endogenous soil nitrate had been consumed by microorganisms in the soil, and no NO was present. NO consumption in the presence of chloramphenicol was then measured by injecting ethanolic solutions of chloramphenicol (between 1 and 2% [vol/vol] final concentration) into the flasks and by adjusting the concentration of NO in the flask headspace to between 3 and 5 parts per million volume (ppmv). Ethanol at concentrations between 1 and 2% did not affect the rate of NO consumption by either soil. The NO concentration was measured at 20-min intervals over a period of 120 to 180 min. NO consumption

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TABLE 1. Enzyme activity in the presence and absence of chloramphenicol and parameters of linear regression equations of enzyme activity versus chloramphenicol concentration

Assay	Soil	Measured activity rate (ng of N g dry soil^{-1} h^{-1}) for chloramphenicol concn (g/liter) of:		Regression equation			
		0	0.1	Intercept	Slope	r^2	P^a
Net nitrite production	Humisol	11.83 (1.46)[b]	10.64 (2.63)	10.13	−2.25	0.569	0.005
	Humisol	3.68 (0.24)	4.57 (0.33)	4.52	−1.02	0.772	<0.005
	Sandy loam	0.35 (0.08)	0.85 (0.06)	0.86	−0.11	0.864	<0.005
Nitrite reduction	Humisol	91.94 (8.23)	35.45 (4.07)	38.49	−5.35	0.361	0.066
	Humisol	30.68 (2.84)	23.61 (5.00)	26.74	2.37	0.121	0.324
	Sandy loam	7.74 (1.99)	0.92 (0.67)	1.30	0.06	0.002	0.910

[a] *P* value for F test of null hypothesis that slope of regression line is 0.
[b] Values in parentheses are standard deviations.

by the humisol and sandy loam soils was a first-order reaction. First-order rate constants were calculated from regression of log NO concentration versus time.

Net production of N_2O from nitrate and N_2O reduction. N_2O production was determined by the DEA assay technique (19). DEA assays were initiated by placing 10 g (fresh weight) of soil and 25 ml of phosphate buffer (50 mM; pH 7) containing 10 mM KNO_3, 10 mM glucose, and chloramphenicol into a series of 150-ml Erlenmeyer flasks. Chloramphenicol concentrations were from 0.1 to 2.0 g/liter. The flasks were evacuated and flushed with N_2 three times and vented to atmospheric pressure. Ten percent of the gas phase over incubated soils was replaced with acetylene to give a final partial pressure of 10 kPa. All incubations were shaken at 200 rpm at room temperature. The time courses of N_2O production were linear over a period of at least 100 min, and the flasks were sampled at 10- to 15-min intervals between 60 and 100 min after the addition of acetylene. Gas samples were stored in 2-ml serum vials with crimped seals (Wheaton, Millville, N.J.).

N_2O consumption was monitored in incubations in which N_2O produced from endogenous soil nitrate was depleted. Flasks containing 10 g of soil and assay solution which was not supplemented with nitrate, chloramphenicol, or acetylene were evacuated, flushed with N_2, and shaken at 200 rpm overnight. The next morning, N_2O produced from endogenous soil nitrate had been consumed by microorganisms in the soil and no N_2O was present. N_2O consumption in the presence of chloramphenicol was then measured by adding ethanolic solutions of chloramphenicol to the flasks and by adjusting the concentration of N_2O in the headspace to 950 ppmv. Ethanol at final concentrations between 1 and 2% (vol/vol) did not affect N_2O consumption by either soil. The decrease in N_2O concentration was measured at 30-min intervals over a period of 150 min. N_2O consumption by the humisol and sandy loam soils was linear over the 150-min period.

Analytical methods. Nitrite was measured by an automated Griess-Ilosvay method (14). Samples for nitrite analysis were collected with a syringe, transferred to 1.5-ml Eppendorf tubes, and centrifuged for 10 min at 15,000 × *g*, and the supernatants were frozen until analysis. Samples for nitrite analysis were diluted 1/10 or 1/50 (vol/vol) before analysis to prevent interference by humic material extracted from the soil. NO was measured with a Sievers chemiluminescence analyzer (model 270B NOA) equipped with an injection port. The N_2O concentration was measured with a Perkin-Elmer gas chromatograph (Porapak Q column; oven temperature, 80°C; detector temperature, 265°C) equipped with a ^{63}Ni electron capture detector (Valco Instruments Co. Inc.).

RESULTS AND DISCUSSION

Net nitrite production from nitrate (nitrate reductase activity). There was a significant linear relationship between the rate of net nitrite production from nitrate and chloramphenicol concentration in both the humisol and sandy loam soils (Table 1 and Fig. 1). The rates of net nitrite production measured in the presence of 0.1 g of chloramphenicol/liter were within ±5% of the values predicted by the regression equations (Table 1). The measured rates of nitrite production in the absence of chloramphenicol were not accurately predicted by the intercept values of the regression equations (Table 1). In two of the three cases the predicted rates were higher than the rates of nitrite production measured in the absence of chloramphenicol (Table 1). The faster nitrite accumulation in the presence of chloramphenicol was probably due to a slowing of nitrite consumption because chloramphenicol prevented synthesis of nitrite reductase enzyme in the 5- or 10-h period during which nitrite production was measured (see below).

Nitrite reduction (nitrite reductase activity). The rate of nitrite consumption was not significantly related to chloram-

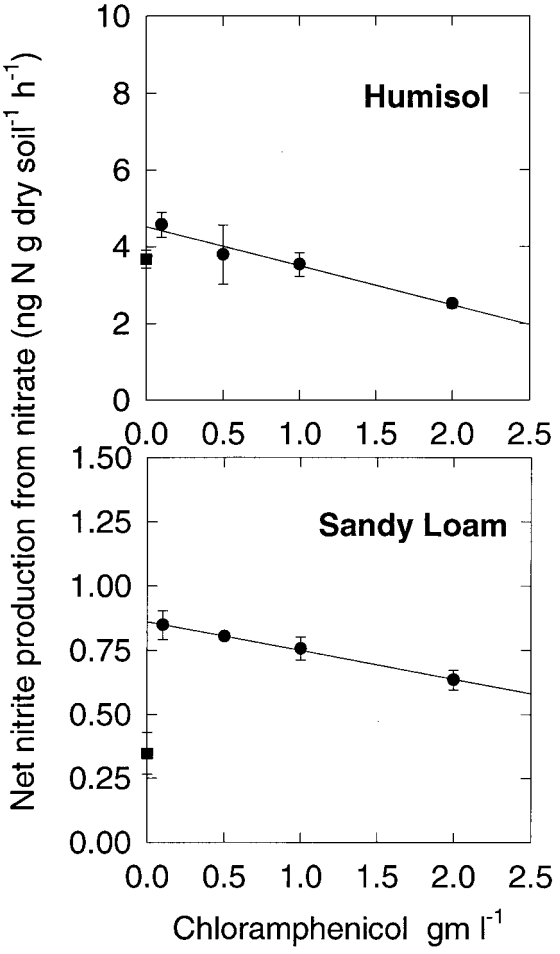

FIG. 1. Net nitrite production from nitrate (nitrate reductase activity) at different chloramphenicol concentrations in humisol and sandy loam soils. The data are the means of triplicate determinations, and the error bars indicate standard deviations that exceed the dimensions of the symbols. Circles, nitrite production rates at the indicated concentrations of chloramphenicol; squares, nitrite production rate in the absence of chloramphenicol. Linear regression parameters are presented in Table 1.

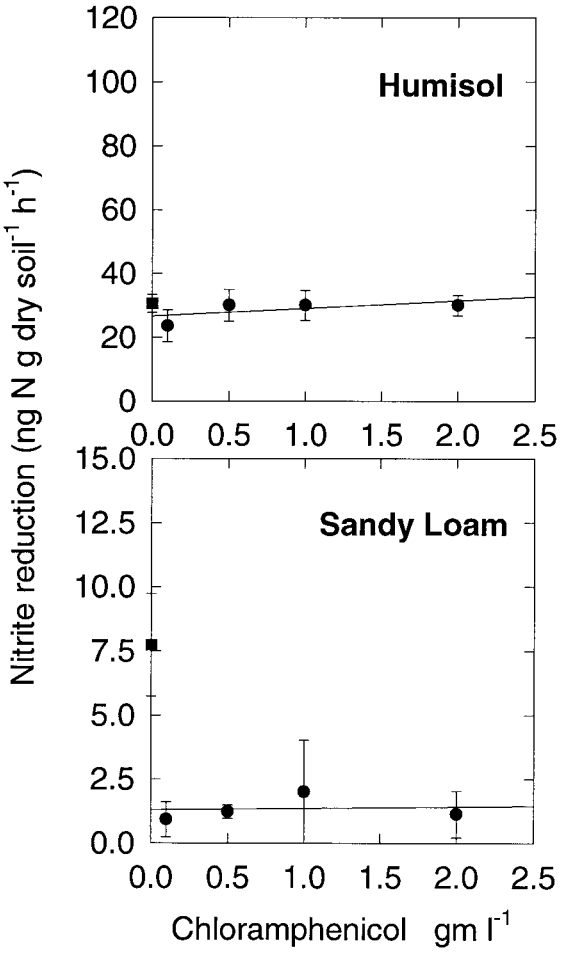

FIG. 2. Nitrite reduction (nitrite reductase activity) at different concentrations of chloramphenicol in humisol and sandy loam soils. The data are the means of triplicate determinations, and the error bars indicate standard deviations that exceed the dimensions of the symbols. Circles, nitrite consumption rates at the indicated concentrations of chloramphenicol; squares, nitrite consumption rate in the absence of chloramphenicol. Linear regression parameters are presented in Table 1.

phenicol concentration (Table 1 and Fig. 2). The measured rates of nitrite consumption in the absence of chloramphenicol were between 1.3 and 8.4 times those measured in the presence of 0.1 g of chloramphenicol/liter (Table 1 and Fig. 2).

The higher nitrite consumption in the absence of chloramphenicol suggests that nitrite reductase was being synthesized, in the absence of chloramphenicol, during the 3- to 6-h assay period. It is reasonable to expect that nitrite reductase could be synthesized during the assay period in the absence of chloramphenicol because nitrite reductase is known to be constitutively produced under aerobic conditions in six strains of denitrifying bacteria (8) and nitrite reductase synthesis in *Pseudomonas stutzeri* is stimulated by oxygen depletion (11). Moreover, Baumann et al. (1, 2) have detected synthesis of nitrite reductase mRNA within 1 h of placing a chemostat culture of "*Pseudomonas denitrificans*" under anaerobic conditions. The inhibition of nitrite consumption by chloramphenicol, especially in the sandy loam (Fig. 2), is consistent with our previous observation of increased nitrite accumulation from nitrate in the presence of chloramphenicol (Fig. 1).

Net production of NO from nitrate (nitrate reductase activity plus nitrite reductase activity). There was a significant linear relationship between the rate of NO production from added nitrate and chloramphenicol concentration (Table 2 and Fig. 3). The measured rates of NO production in the presence of 0.1 g of chloramphenicol/liter were within ±3% of the intercept values predicted by the regression equations (Table 2). The observed decreases in the rates of production of NO from nitrate were strongly related to chloramphenicol concentration (P values of <0.001 [Table 2]) and are consistent with our observation of the inhibition of existing nitrate reductase enzymes by chloramphenicol (Fig. 1). The measured rates of NO production in the absence of chloramphenicol were between 1.3 and 1.7 times the rates predicted from the intercept values of the regression equations (Table 2 and Fig. 3).

NO reduction to N_2O (NO reductase activity). The first-order rate constants of NO consumption were not significantly related to chloramphenicol concentration (Table 2 and Fig. 4). The measured rate of NO consumption in the absence of chloramphenicol was approximately 1.1 to 1.2 times that predicted by the intercept values of the regression equations (Fig. 4).

Net production of N_2O from nitrate (nitrate reductase plus nitrite reductase plus NO reductase activity; DEA). There was a significant linear relationship between the rate of N_2O production from nitrate and chloramphenicol concentration (Table 3 and Fig. 5). The inhibition of N_2O production from nitrate by increasing chloramphenicol concentration is consistent with our observation of the inhibition of existing nitrate reductase enzymes by chloramphenicol (Fig. 1). The measured N_2O production in the presence of 0.1 g of chloramphenicol/liter was within ±5% of the intercept values predicted by the regression equations. The measured N_2O production in the

TABLE 2. Enzyme activity in the presence and absence of chloramphenicol and parameters of linear regression equations of enzyme activity versus chloramphenicol concentration

Assay	Soil	Measured activity rates[a] for chloramphenicol concn (g/liter) of:		Regression equation			
		0	0.1	Intercept	Slope	r^2	P^b
Net NO production	Humisol	2.74 (0.08)[b,c]	2.03 (0.22)	1.97	−0.03	0.688	0.001
	Humisol	2.91 (0.09)	2.14 (0.04)	2.18	−0.24	0.947	<0.005
	Sandy loam	1.47 (0.13)	0.87 (0.05)	0.89	−0.18	0.952	<0.0005
NO reduction	Humisol	0.026 (0.001)	0.022 (0.001)	0.024	−6.28	0.183	0.254
	Humisol	0.026 (0.002)	0.024 (0.0007)	0.022	4.37	0.162	0.194
	Sandy loam	0.018 (0.0005)	0.016 (0.0006)	0.016	−0.0003	0.58	0.449

[a] Rates for net NO production are in nanograms of N per gram of dry soil per minute; rates for NO reduction are first-order rate constant k (per minute).
[b] P value for F test of null hypothesis that slope of regression line is 0.
[c] Values in parentheses are standard deviations.

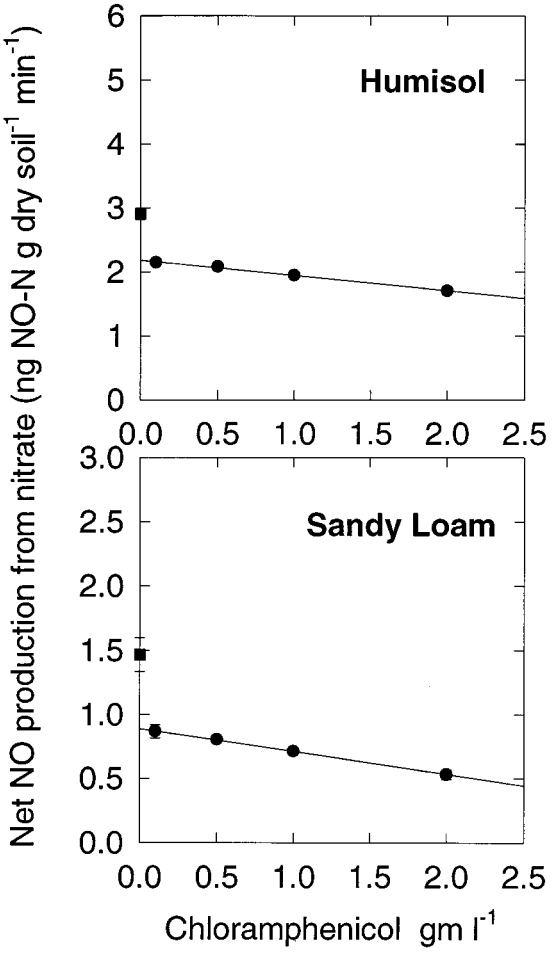

FIG. 3. Net NO production from nitrate (nitrate reductase plus nitrite reductase activity) versus chloramphenicol concentration in humisol and sandy loam soils. The data are the means of triplicate determinations, and the error bars indicate standard deviations that exceed the dimensions of the symbols. Circles, NO production rates at the indicated concentrations of chloramphenicol; squares, NO production rate in the absence of chloramphenicol. Linear regression parameters are presented in Table 2.

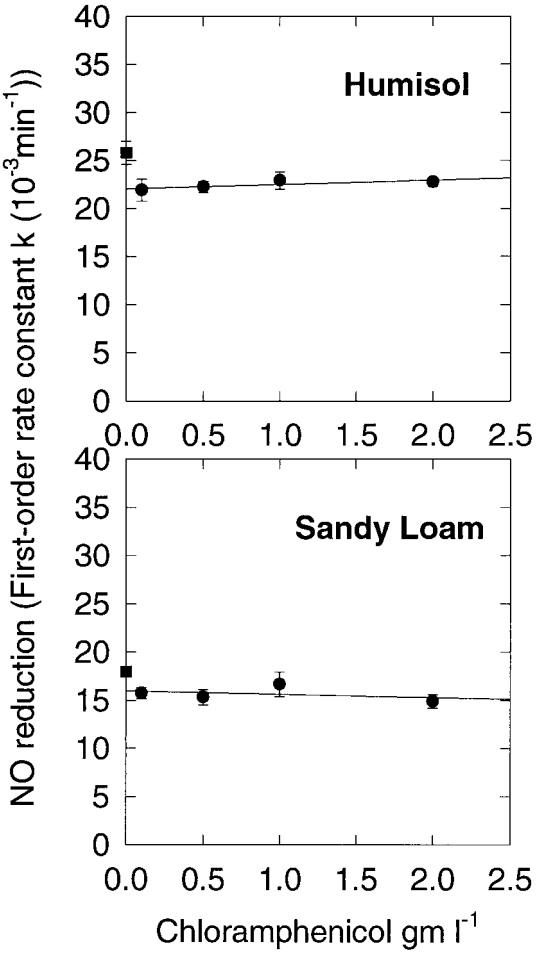

FIG. 4. First-order rate constants for NO reduction to nitrous oxide (NO reductase activity) versus chloramphenicol concentration in humisol and sandy loam soils. The data are the means of triplicate determinations, and the error bars indicate standard deviations that exceed the dimensions of the symbols. Circles, NO consumption rates at the indicated concentrations of chloramphenicol; squares, NO consumption rate in the absence of chloramphenicol. Linear regression parameters are presented in Table 2.

absence of chloramphenicol was 1.2 to 1.8 times that predicted from the intercept values of the regression equations (Table 3).

Reduction of N_2O (N_2O reductase activity). There was a significant linear relationship between the N_2O consumption rate and the chloramphenicol concentration in the sandy loam soil but not in the humisol (Table 3 and Fig. 6). The measured rate of N_2O consumption in the presence of 0.1 g of chloramphenicol/liter was within ±9.0% of the rate predicted by the regression equation. The measured rates of N_2O consumption in the absence of chloramphenicol were between 1.2 and 1.3 times those predicted from the intercept values of the regression equations (Table 3 and Fig. 6). N_2O reductase activity is not measured during DEA assays because N_2O consumption is blocked by acetylene (9, 10) during the assay, and N_2O accumulation is used as the index of denitrification activity.

Net production and reduction rates. It is not possible to directly compare rates of nitrite, NO, and N_2O consumption between assays because long incubation times (during which bacterial growth probably occurred) were required to deplete existing pools of soil nitrate, NO, or N_2O before consumption assays could be initiated. Moreover, rates of N_2O production (DEA) cannot be directly compared to rates of nitrite and NO production because the rate of N_2O production in the presence of acetylene represents the total reduction of nitrate to N_2O while the rates of nitrite and NO production represent the transient accumulation of nitrite and NO in excess of that undergoing reduction to N_2O during the assay period.

Chloramphenicol and the synthesis of DEA enzymes. The measured rates of net production and reduction of nitrite, NO, and N_2O in the absence of chloramphenicol were not accurately predicted by the intercept values of regression equations of rate versus chloramphenicol concentration (Tables 1 to 3 and Fig. 1 to 6). The inability of the regression equations to predict the measured enzyme activities at 0 g of chloramphenicol/liter could be caused by the synthesis of new enzymes during the assay period in the absence of chloramphenicol, or a portion of the total denitrifying population could be extremely sensitive to low concentrations of chloramphenicol and exhibit a concentration-independent response at low chloramphenicol concentrations.

While our data cannot rule out the possibility of a concentration-independent effect at low chloramphenicol concentrations (a possibility relevant to all inhibitor studies with mixed

TABLE 3. Enzyme activity in the presence and absence of chloramphenicol and parameters of linear regression equations of enzyme activity versus chloramphenicol concentration

Assay	Soil	Measured activity rates (ng of N g dry soil^{-1} min^{-1}) for chloramphenicol concn (g/liter) of:		Regression equation			
		0	0.1	Intercept	Slope	r^2	P^a
DEA (net N_2O production)	Humisol	50.49 (6.44)[b]	40.90 (0.44)	41.54	−5.47	0.572	0.018
	Humisol	30.96 (4.13)	22.63 (4.35)	21.77	−6.91	0.654	0.003
	Sandy loam	9.37 (1.65)	5.58 (0.41)	5.31	−0.17	0.737	0.002
N_2O reduction	Humisol	119.81 (2.84)	100.98 (2.62)	102.67	−4.26	0.194	0.152
	Sandy loam	130.62 (10.58)	90.34 (7.93)	99.10	−9.39	0.464	0.015

[a] P value for F test of null hypothesis that slope of regression line is 0.
[b] Values in parentheses are standard deviations.

populations), one would expect that synthesis of denitrifying enzymes would occur in anaerobic incubations of soil supplemented with glucose and nitrate because synthesis of mRNAs for denitrifying enzymes is known to occur within 1 h of placing a pure culture of "*P. denitrificans*" under anaerobic conditions (1) and the patterns of N_2O accumulation from long term soil DEA assays carried out without chloramphenicol are consistent with populations undergoing exponential growth (17). We suggest that 0.1 g of chloramphenicol/liter should be used in DEA assays to preclude the possibility of denitrifying enzyme synthesis.

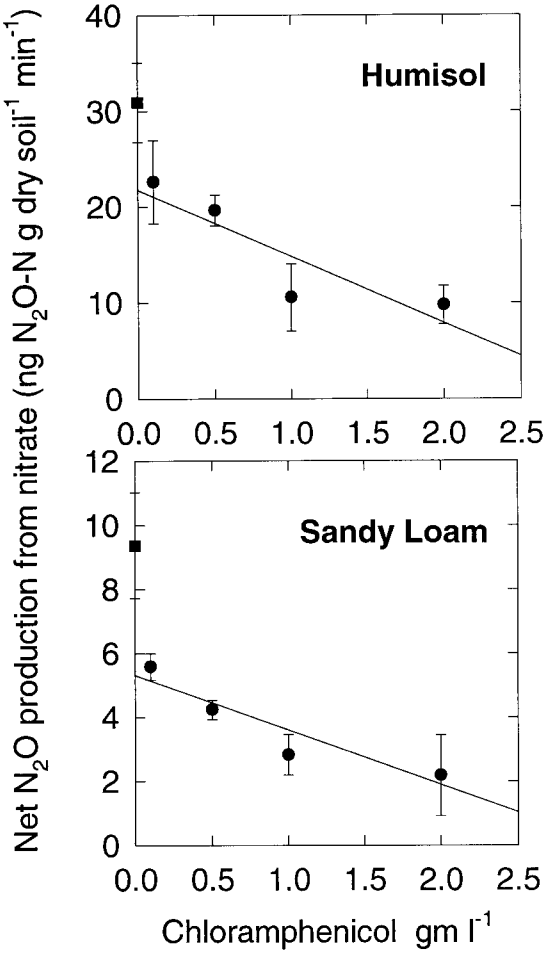

FIG. 5. Net N_2O production from nitrate (nitrate reductase plus nitrite reductase plus NO reductase activity) versus chloramphenicol concentration in humisol and sandy loam soils. The data are the means of triplicate determinations, and the error bars indicate standard deviations that exceed the dimensions of the symbols. Circles, N_2O production rates at the indicated concentrations of chloramphenicol; squares, N_2O production rate in the absence of chloramphenicol. Linear regression parameters are presented in Table 3.

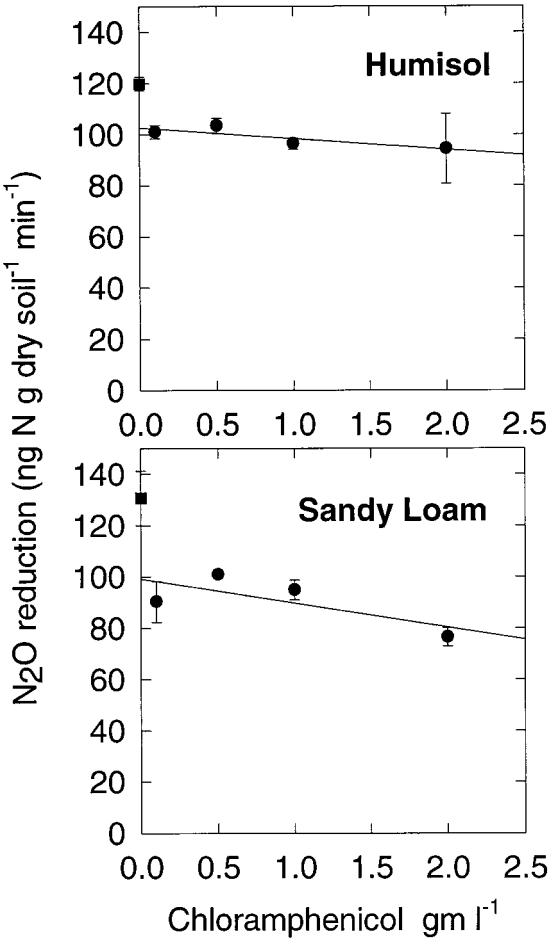

FIG. 6. N_2O reduction (nitrous oxide reductase activity) versus chloramphenicol concentration in humisol and sandy loam soils. The data are the means of triplicate determinations, and the error bars indicate standard deviations that exceed the dimensions of the symbols. Circles, nitrous oxide consumption rates at the indicated concentrations of chloramphenicol; squares, nitrous oxide consumption rate in the absence of chloramphenicol. Linear regression parameters are presented in Table 3.

Chloramphenicol and the inhibition of existing denitrifying enzymes. Chloramphenicol at concentrations greater than 0.1 g/liter reduced the activity of nitrate reductase enzymes in both soils (Table 1 and Fig. 1) and that of N_2O reductase enzymes in the sandy loam soil (Table 3 and Fig. 6). When the concentration of chloramphenicol was increased from 0.1 to 2.0 g/liter (0.3 to 6.0 mM), the rate of nitrite production from nitrate decreased by 25 to 46%. The rate of NO production from nitrate decreased by 20 to 39%, and the rate of N_2O production from nitrate (DEA) decreased by 21 to 61% (Tables 1 to 3 and Fig. 1, 3, and 5). The decrease in enzyme activity caused by chloramphenicol is most likely the result of the disruption of previously existing nitrate reductase enzymes, because we used a chloramphenicol concentration in excess of the 0.3- to 5.0-μM concentration known to inhibit protein synthesis in cultures of gram-positive and gram-negative bacteria (12) and chloramphenicol within the 0.1- to 2.0-g/liter concentration range is known to disrupt the activity of existing nitrate reductase enzymes in pure cultures of both *F. canadensis* and "*P. denitrificans*" and in cell extracts of "*P. denitrificans*" (23). Moreover, we did not observe a chloramphenicol concentration effect on the activity of nitrite reductase or NO reductase enzymes. We suggest that chloramphenicol at concentrations greater than 0.1 g/liter can disrupt the activity of previously existing nitrate reductase enzymes in the two soils studied and should not be used in DEA assays.

Modifications to the DEA assay. Pell et al. (17) proposed two possible methods for estimating DEA which would avoid the problem of chloramphenicol inhibition of existing denitrifying enzymes. The growth-associated product formation method involves fitting a series of timed measurements of N_2O accumulation in the absence of chloramphenicol to a product formation equation and solving the fitted equation for the initial denitrification rate (20). While the growth-associated product formation approach is of interest and holds out the possibility of obtaining the in situ growth rate of denitrifying organisms, the long incubation times required (between 380 and 600 min) would likely preclude the use of the technique for large numbers of replicate samples. Moreover, assumptions underlying the technique, particularly the assumptions that the denitrifying organisms are undergoing balanced growth and that specific enzyme activity remains constant during the incubation period (20), need to be verified for indigenous denitrifying organisms growing in soil slurries.

The second method proposed by Pell et al. (17) involves measuring DEA in the presence of a range of chloramphenicol concentrations and extrapolating the N_2O production rate versus chloramphenicol concentration data to the rate at 0 g of chloramphenicol/liter. We used this method to investigate the influence of chloramphenicol on the activity of the enzymes associated with each step in the denitrification process. Enzyme rate versus chloramphenicol concentration was measured by using triplicate samples to characterize the variability of enzyme activity in soil. In all cases where there was a significant relationship between enzyme activity and chloramphenicol concentration, the activity at 0 g of chloramphenicol/liter predicted from regression equations was within a few percent of the value measured in the presence of 0.1 g of chloramphenicol/liter (Tables 1 to 3 and Fig. 1, 3, 5, and 6).

A concentration of 0.1 g of chloramphenicol/liter appears to be high enough to prevent the synthesis of denitrifying enzymes during the DEA assay period and low enough not to disrupt existing denitrifying enzymes by more than a few percent. We suggest that DEA assays can be carried out with a single (0.1-g/liter) chloramphenicol concentration and that using a series of chloramphenicol concentrations would improve the estimate only slightly while substantially increasing the time and effort required to obtain a DEA measurement.

ACKNOWLEDGMENTS

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