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Inhibition of Existing Denitrification Enzyme Activity by Chloramphenicol

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Chloramphenicol completely inhibited the activity of existing denitrification enzymes in acetylene-block incubations with (i) sediments from a nitrate-contaminated aquifer and (ii) a continuous culture of denitrifying groundwater bacteria. Control flasks with no antibiotic produced significant amounts of nitrous oxide in the same time period. Amendment with chloramphenicol after nitrous oxide production had begun resulted in a significant decrease in the rate of nitrous oxide production. Chloramphenicol also decreased (>50%) the activity of existing denitrification enzymes in pure cultures of *Pseudomonas denitrificans* that were harvested during log-phase growth and maintained for 2 weeks in a starvation medium lacking electron donor. Short-term time courses of nitrate consumption and nitrous oxide production in the presence of acetylene with *P. denitrificans* undergoing carbon starvation were performed under optimal conditions designed to mimic denitrification enzyme activity assays used with soils. Time courses were linear for both chloramphenicol and control flasks, and rate estimates for the two treatments were significantly different at the 95% confidence level. Complete or partial inhibition of existing enzyme activity is not consistent with the current understanding of the mode of action of chloramphenicol or current practice, in which the compound is frequently employed to inhibit de novo protein synthesis during the course of microbial activity assays. The results of this study demonstrate that chloramphenicol amendment can inhibit the activity of existing denitrification enzymes and suggest that caution is needed in the design and interpretation of denitrification activity assays in which chloramphenicol is used to prevent new protein synthesis.

Chloramphenicol amendment is often used to prevent de novo protein synthesis during microbial activity assays. New protein synthesis and growth may occur in response to favorable conditions, created either intentionally or inadvertently, in an activity assay medium, and this growth can complicate interpretation of assay results. Chloramphenicol is a broad-spectrum bacteriostatic agent that inhibits new protein synthesis by binding to ribosomes (21), and amendment with this compound is part of a recommended protocol for measuring denitrification enzyme activity (DEA) in soils (30). This protocol is not designed to measure in situ denitrification activity; instead, it provides a relative measure of the existing denitrification enzyme content of a given environment. Factors that affect the activity of the enzymes (oxygen, organic carbon, and nitrate) are optimized for denitrification, and chloramphenicol is added to prevent new enzyme production. Investigators have used this protocol to investigate phases of and changes in the gaseous products of denitrification following onset of anaerobiosis in soil (6, 25), to examine the effects of soil drying on enzyme activity (24), to identify sources of N_2O following wetting of dry soil (23), to develop stochastic models of soil denitrification (19), and to examine the predictive utility of DEA with regard to actual field denitrification rates (9, 15, 20). An important assumption that is made when chloramphenicol is used is that existing enzyme activity is not affected. This assumption has rarely, if ever, been tested.

In this study, we attempted to measure DEA associated with sediments obtained from a nitrate-contaminated aquifer. Incubation flasks were flushed with N_2 to remove O_2 and amended with KNO_3 and glucose to eliminate the possibility of nitrate or organic carbon limitation. Chloramphenicol was added to prevent new denitrification enzyme synthesis in response to the incubation conditions that favored growth. In essence, this procedure was an application of the soil DEA assay to a different type of environmental sample. After several unsuccessful attempts at measuring DEA by using aquifer sediments obtained from previously identified denitrifying zones, we conducted experiments in an effort to explain this anomalous result. We report here results, obtained with environmental samples and laboratory cultures, that demonstrate that chloramphenicol amendment can inhibit the activity of existing denitrification enzymes. This finding has important implications for studies of denitrification that use DEA assays employing chloramphenicol.

MATERIALS AND METHODS

Incubations with aquifer sediments. Aquifer sediments and water samples were obtained from a nitrate-contaminated, shallow, sand and gravel aquifer located on Cape Cod, Mass. Contamination at this site is the result of over 50 years of land disposal of secondarily treated sewage (13), and the contaminated portion of the aquifer is the focus of an ongoing interdisciplinary research program (8). Previous work at this site has established that biological denitrification is the primary electron-accepting process in at least a portion of the contaminated aquifer (27, 28), and the sediments and water used in this study were collected from a zone exhibiting maximal denitrification activity (3.7 m below water table, site F473 [28]).

Water samples from the contaminant plume were collected from multilevel samplers (14) by using a peristaltic pump. The samples were pumped into 1-liter glass bottles that were overfilled with at least 3 liters of sample and then

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capped; care was taken to minimize atmospheric contamination. Water samples were stored at 4°C. Aquifer sediments were collected with a wireline piston corer (33) in conjunction with hollow-stem auger drilling. A center bit was inserted inside the auger flights during the augering process to prevent aquifer material from entering the hollow stem prior to core collection. Sediment cores were collected in 5.1-cm-diameter aluminum liners, capped, and stored at 4°C until used. Incubations with groundwater and aquifer sediments were begun within 24 h of collection.

Denitrification activity associated with aquifer sediments was quantified with sediment-slurry incubations by the acetylene-block technique (1, 32). Subsamples (45 g [wet weight]) of homogenized aquifer sediment and 50 ml of aquifer water were added to 125-ml wide-mouth Erlenmeyer flasks in an anaerobic glove bag. Chloramphenicol was added (0.5 ml of an anoxic aqueous stock solution; final concentration, 300 mg/liter) at either the outset of the incubation or after N_2O production had begun. Control flasks received 0.5 ml of anoxic, sterile, deionized water. Flasks were stoppered, the stoppers were fastened in place with vinyl tape, and the flasks were flushed with O_2-free N_2 for 30 min. Flasks were then amended with 15 ml of C_2H_2 (86 kPa, 25°C) and incubated in a water bath shaker at 12°C. Headspace samples were withdrawn periodically for N_2O determination. All treatments were performed in quadruplicate flasks.

Chemostat experiments. A denitrifying, continuous culture of free-living groundwater bacteria was established and maintained in a chemostat with an inoculum of groundwater collected from a denitrifying zone in the contaminant plume. The feed solution was (0.2-μm-pore-size) filter-sterilized groundwater obtained from a deeper zone in the contaminant plume, which was handled to minimize exposure to atmospheric O_2. The feed solution dissolved organic carbon (DOC) was 2 mg of C per liter, and the NO_3^- concentration was adjusted to 2 mg of N per liter by adding a sterile NO_3^- stock solution. Feed solution was introduced into the culture flask, by using a metering peristaltic pump, at a dilution rate of 0.005 h^{-1}. The dilution rate was chosen to closely match previously determined growth rates for unattached bacteria in the contaminant plume (10). The headspaces of the culture flask and feed solution jar were connected to a tank of O_2-free N_2 with copper tubing and sterile (0.1-μm-pore-size) filters. A constant pressure of 97 kPa was maintained in each vessel. The culture was continuously stirred with a magnetic stir bar, and the culture flask was immersed in a water bath at 18°C. Samples for NO_3^-, NO_2^-, and DOC determination and denitrification activity assay were collected by syringe with a sterile needle inserted through a sampling port septum. Samples for NO_3^- and NO_2^- determination were filtered (0.45-μm-pore-size filter) and frozen; samples for DOC determination were filtered through precombusted (300°C) glass-fiber filters (Whatman GF/C) into precombusted glass bottles and stored at 4°C.

The effect of chloramphenicol on denitrification activity in the chemostat culture was measured by acetylene-block experiments. Chloramphenicol (4.5 mg) was added to 30-ml serum bottles, and the bottles were sealed with butyl rubber stoppers. The final antibiotic concentration was 300 mg/liter; control bottles received no antibiotic. The bottles were flushed with O_2-free N_2 for 30 min, and then 15 ml of chemostat culture was injected into each bottle. Each bottle then received 5 ml of C_2H_2 (86 kPa, 25°C), the bottles were incubated at 18°C, and headspace samples were withdrawn for N_2O determination at 0, 4, 10.5, 21, 32.5, and 75 h.

Carbon starvation experiments. Experiments were performed with pure cultures of *Pseudomonas denitrificans* (ATCC 13867) that were undergoing carbon starvation. The cultures were grown anaerobically to late-exponential phase in a medium having the following composition: yeast extract, 5.0 g; $MgSO_4$, 0.2 g; $NaNH_4HPO_4 \cdot 4H_2O$, 3.5 g; K_2HPO_4, 5.0 g; KH_2PO_4, 3.9 g; $FeCl_3$, 0.006 g; KNO_3, 1.01 g; and distilled water, 1,000 ml. The cultures were harvested by centrifugation and washed with anaerobic, sterile salts solution (growth medium without yeast extract, $FeCl_3$, and KNO_3). After two washings, subsamples of the cell suspensions were transferred to 2,300-ml Fernbach flasks containing 2,000 ml of starvation medium to achieve a starting optical density at 660 nm (OD) of 0.05. The starvation medium composition was identical to that of the growth medium, except that the starvation medium contained 0.05 g of KNO_3 (500 μM) per liter and no yeast extract. The cultures were maintained in an anaerobic glove bag at room temperature and were continuously stirred.

Biomass in the starvation cultures was monitored by periodically measuring the OD and the number of CFU. The effect of chloramphenicol on denitrification enzyme activity in the starvation cultures was measured with two different assays: (i) NO_3^- consumption and (ii) N_2O production in the presence of acetylene. Assays were short-term incubations lasting no longer than 150 min. Nitrate consumption assays were performed by transferring 50 ml of starvation culture into sterile 125-ml Erlenmeyer flasks. Treatments were done in duplicate flasks and consisted of amendments of 5 ml of sterile, anaerobic chloramphenicol stock (final concentration, 300 mg/liter) or 5 ml of sterile, anaerobic water. After 30 min of contact time, 1 ml of sterile, anaerobic yeast extract stock (final concentration, 250 mg/liter) was added to each flask and T_0 samples were taken. For the next 120 min, 3 ml of subsample was removed every 30 min with a sterile pipet, filtered (0.45-μm-pore-size filter), and frozen for NO_3^- determination. Nitrous oxide production assays were performed by injecting 15 ml of starvation culture into a 30-ml serum bottle which had previously been sealed in the glove bag with a butyl rubber stopper. The bottle headspace was vented with a needle during injection in order to maintain ambient atmospheric pressure in the bottle. Treatments were done in triplicate and consisted of amendment with 1.5 ml of sterile, anaerobic chloramphenicol stock (final concentration, 300 mg/liter) or 1.5 ml of sterile, anaerobic water. After 30 min of contact time, all bottles received 1.5 ml of sterile, anaerobic yeast extract stock (final concentration, 250 mg/liter) and 5 ml of acetylene (86 kPa, 25°C). Bottles were placed in an incubator-shaker (25°C), and headspace samples were withdrawn periodically for N_2O determination.

Analytical techniques. Nitrous oxide was measured with a gas chromatograph (model 301; HNU Systems, Newton, Mass.) with two 0.3-cm-diameter stainless steel columns packed with Porapak N and connected in series to a six-port switching valve. The valve allowed for backflushing of the first column to prevent acetylene from entering the detector. The chromatograph was equipped with an electron capture detector (model 140 BN; Valco Instruments Co., Houston, Tex.) operating at 240°C. The carrier gas was 5% CH_4 in argon at a flow rate of 40 ml/min, and the oven temperature was 90°C. The instrument was calibrated with standard gas mixtures (Scott Specialty Gases, Plumsteadville, Pa.). Aqueous concentrations of N_2O in the incubation flasks were calculated by using Bunsen absorption coefficients (31). Nitrate and nitrite were measured colorimetrically with a

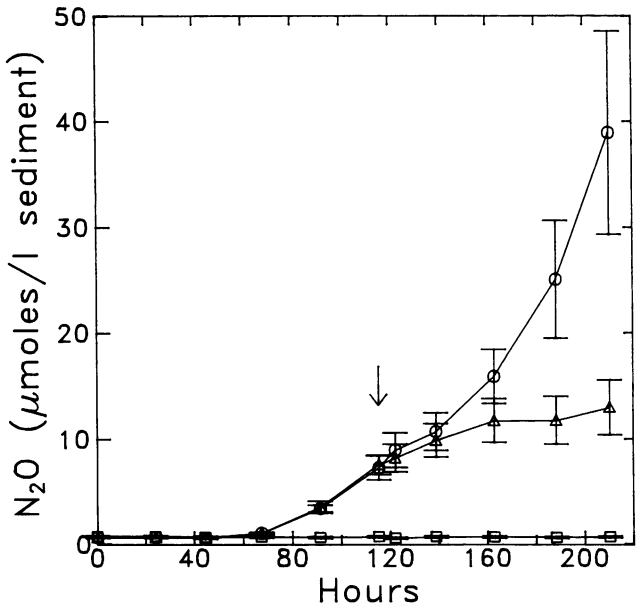

FIG. 1. Time course of nitrous oxide production for acetylene-block sediment-slurry incubations. Aquifer sediment and water were collected from a previously identified denitrifying zone. Incubations were performed at 12°C by using ambient organic carbon and nitrate. Incubations were not amended with chloramphenicol (○), amended at the outset of the incubation (□), or amended at 115 h (△). The chloramphenicol concentration was 300 mg/liter. Error bars represent ±1 standard error of the mean for four replicate flasks.

flow-injection autoanalyzer (Lachat Instruments, Mequon, Wis.) by the cadmium reduction technique (7). Chloramphenicol caused a negative analytical interference for NO_3^- by interfering with the reduction of NO_3^- to NO_2^-. NO_3^- concentrations for samples that contained chloramphenicol were corrected accordingly. DOC was quantified with a total organic carbon analyzer (model 700; Oceanographic International, College Station, Tex.) by the wet persulfate oxidation method (16). Ammonium was quantified colorimetrically with the Nessler reagent (Hach Co., Loveland, Colo.). Chloramphenicol was obtained from Sigma Chemical (St. Louis, Mo.) (two separate lots) and Aldrich Chemical Co. (Milwaukee, Wis.).

RESULTS

Incubations with aquifer sediments. When sediments were collected from a previously identified denitrifying zone in a groundwater contaminant plume and incubated by an C_2H_2-block sediment-slurry technique, chloramphenicol amendment prevented N_2O accumulation throughout a 9-day time course (Fig. 1). Control flasks (no chloramphenicol present) showed an increase of nearly 40 μmol of N_2O · liter of sediment^{-1} during the same time period (Fig. 1). The incubations were performed without addition of nitrate or organic carbon and at ambient groundwater temperature (12°C). A lag period (>70 h) was observed in the control flasks before N_2O production began (Fig. 1). The difference between control and chloramphenicol treatments was not necessarily caused by new enzyme synthesis during the incubation; when chloramphenicol was added to a third set of flasks after N_2O production had begun (115 h), the rate of N_2O production was significantly reduced (Fig. 1). Results shown in Fig. 1 are very typical for chloramphenicol-amended C_2H_2-block incubations performed with aquifer sediments from the Cape Cod site; similar results have been obtained for several locations in the contaminant plume (data not shown). In addition, incubations designed to measure consumption of NO_3^-, NO_2^-, or N_2O under nonlimiting conditions (no O_2 and excess electron donor and acceptor) in the presence of chloramphenicol also resulted in no measurable DEA (data not shown).

TABLE 1. Composition of groundwater chemostat feed solution and effluent

Sample	Concn (mg of N/liter)		DOC concn (mg of C/liter)
	Nitrate	Nitrite	
Feed solution	2.20	ND[a]	2.0
Chemostat effluent	1.41	0.48	2.1

[a] ND, below the limit of detection (0.02 mg of N per liter).

Chemostat experiments. A continuous culture of denitrifying groundwater bacteria was established and maintained in a chemostat with an inoculum of groundwater obtained from the contaminant plume. This culture provided a source of denitrifying contaminant plume bacteria living under a nutrient regime that closely mimicked that found in situ. This denitrifying culture was maintained in the chemostat for 119 days without washout occurring. Nitrate was consumed and nitrite was produced by the chemostat culture (Table 1), but no significant consumption of dissolved organic carbon was evident (Table 1). Ammonium was not detected in the feed solution or the effluent (limit of detection, 0.1 mg of N per liter).

Chloramphenicol amendment also prevented N_2O production in acetylene-block incubations with samples from the groundwater chemostat (Fig. 2). The rate of N_2O production

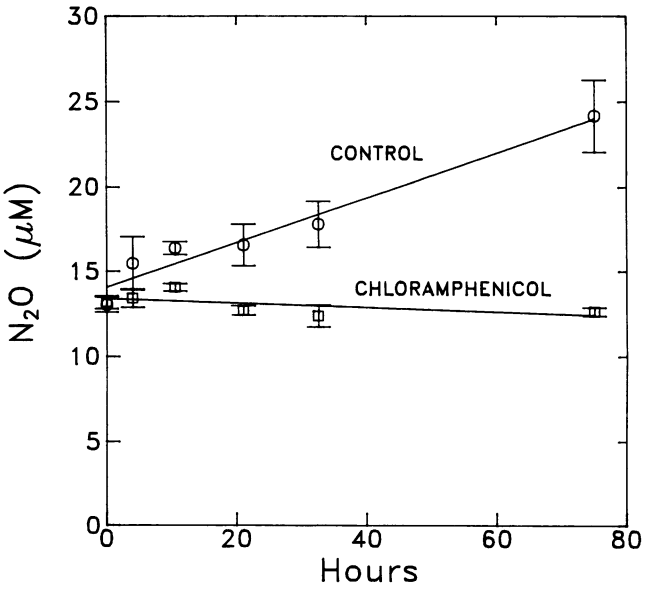

FIG. 2. Time course of nitrous oxide production from an acetylene-block incubation with a groundwater chemostat culture. Incubation temperature was 18°C, and the chloramphenicol concentration was 300 mg/liter. Error bars represent ±1 standard error of the mean for four replicate flasks. Lines are linear regression fits.

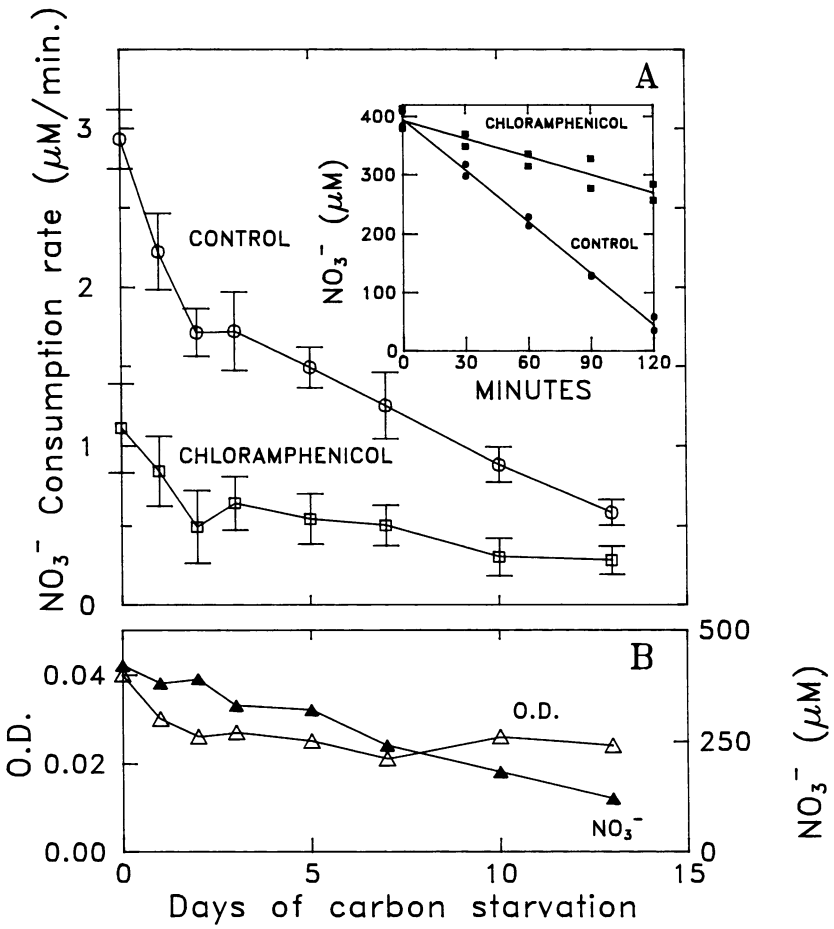

FIG. 3. (A) Rates of NO_3^- consumption versus time of starvation for *P. denitrificans* undergoing carbon starvation; error bars represent 95% confidence intervals for the calculated rates. The time course of NO_3^- consumption for day-0 assays is shown in the inset; the lines are linear regression fits. (B) OD and NO_3^- concentrations in the starvation culture versus time of starvation.

for antibiotic-amended bottles was not significantly different from zero ($P < 0.01$). Control incubations showed a linear ($r^2 = 0.59$) increase in N_2O, with no lag in N_2O production (Fig. 2). These incubations were performed without altering the organic carbon and NO_3^- concentrations in the chemostat samples. The rate of N_2O increase for the control flasks was 132 nmol · liter of culture^{-1} · h^{-1}. The 95% confidence interval for this rate estimate was ±49 nmol · liter of culture^{-1} · h^{-1}. This rate corresponds to an NO_3^- consumption of 264 nmol · liter of culture^{-1} · h^{-1}, because of the 2:1 stoichiometry of NO_3^- reduction to N_2O, and represents 94% of the total NO_3^- consumption in the chemostat (282 nmol · liter of culture^{-1} · h^{-1}) calculated by using the data in Table 1 and the chemostat dilution rate. It should be noted that a significant portion of the NO_3^- consumed in the chemostat was reduced only to NO_2^- (Table 1), and this single-step reduction would not be apparent in the C_2H_2-block assay.

Carbon starvation experiments. Chloramphenicol amendment significantly lowered nitrate consumption rates in short-term incubations with samples of a denitrifying culture of *P. denitrificans* that was experiencing carbon starvation (Fig. 3A). Nitrate consumption incubations with subsamples of the starvation culture were performed on eight occasions during the course of a 13-day carbon starvation experiment. On all occasions, the rate of NO_3^- consumption in the chloramphenicol-amended flasks was 50% or less of the rate of NO_3^- consumption in the control flasks (Fig. 3A). The differences between treatment rates were significant at the 95% confidence level (Fig. 3A). It is important to note that the incubation conditions used to measure these rates included the addition of a yeast extract stock solution to the assay flasks. Therefore, the measured rates represent the potential DEA in the starvation culture upon relief of carbon limitation but do not represent the endogenous rate of denitrification during starvation. Denitrification rates were calculated from five time points during a 120-min incubation. The time courses used to calculate the rates for day 0 are shown in the inset of Fig. 3A. Linear decreases in NO_3^- concentration were observed for both the chloramphenicol ($r^2 = 0.92$) and control ($r^2 = 0.99$) flasks, and these time courses are representative of all eight of the rate determinations which are depicted in Fig. 3A. OD of the starvation culture decreased from 0.040 to 0.024 during the 13-day carbon starvation period, while the nitrate concentration in the culture decreased from 420 to 120 μM in the same time period (Fig. 3B). The relationship between the OD and the number of CFUs, determined in a separate starvation experiment, was as follows: CFU = $[(1.85 \times 10^9) \times \text{OD}] - (1.84 \times 10^7)$.

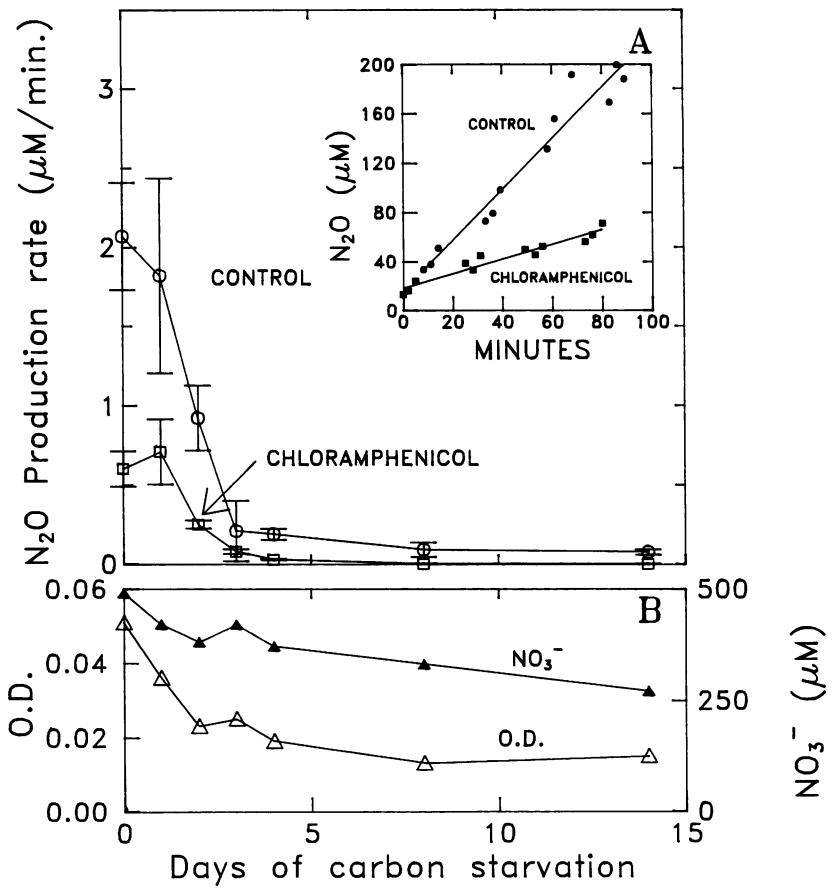

FIG. 4. (A) Rates of N_2O production versus time of starvation for *P. denitrificans* undergoing carbon starvation; error bars represent 95% confidence intervals for the calculated rates. The time course of N_2O production for day-0 assays is shown in the inset; the lines are linear regression fits. (B) OD and NO_3^- concentrations in the starvation culture versus time of starvation.

Addition of chloramphenicol to acetylene-block incubations with *P. denitrificans* in a parallel carbon starvation experiment lowered rates of N_2O production relative to those of controls (Fig. 4A). Differences in N_2O production rates between the two treatments were always twofold or greater and were significant at the 95% confidence level on all occasions, except for day 3 (Fig. 4A). Again, it should be noted that the incubations used for determining the rates shown in Fig. 4A involved relief of carbon limitation and represent potential, not actual, denitrification activity in the starvation culture. Time courses of N_2O production used for the rate determinations on day 0 are shown in the inset of Fig. 4A. N_2O production was linear for both chloramphenicol ($r^2 = 0.93$) and control treatments ($r^2 = 0.95$), and this result was typical for all of the N_2O production time courses which were performed. Time courses for the rate determinations never exceeded 150 min. The OD of the starvation culture decreased from 0.051 to 0.015 during the 14-day carbon starvation period, while the nitrate concentration in the culture decreased from 490 to 270 μM in the same time period (Fig. 4B).

DISCUSSION

We attempted to measure DEA associated with aquifer sediments obtained from an NO_3^--contaminated aquifer. In all instances, our results indicated that there was no DEA in the aquifer. Despite this result, there is a great deal of evidence that clearly indicates that denitrification was occurring in the aquifer. Sediments and water from numerous sites within the contaminant plume display denitrification potential (in the absence of chloramphenicol), and the magnitude of the expressed potentials correlates with availability of NO_3^- in the contaminated portion of the aquifer (27). The geochemistry of the contaminant plume has been substantially modified by the activity of denitrifying bacteria. A zone of elevated N_2O and excess dissolved inorganic carbon and N_2 relative to atmospheric saturation indicate that NO_3^- is being used to oxidize the available organic carbon in parts of the contaminated portion of the aquifer (28). Isotopic fractionation, consistent with that expected for denitrification, has been observed for the $\delta^{15}N$ of NO_3^- and N_2 (28) and provides further evidence that active denitrification is occurring in this system. Additionally, an in situ tracer test (as described in reference 29) with aquifer water amended with a conservative tracer and C_2H_2 resulted in in situ production of N_2O, the peak concentration of which coincided with elevated concentrations of the conservative tracer (26). This evidence caused us to doubt either our application of or the actual validity of the DEA assay in this environment. In the process of testing possible problems with our techniques for measuring DEA, we conducted a series of experiments examining the effects of chloramphenicol on DEA.

Chloramphenicol completely inhibited N_2O production in

C_2H_2-block incubations with aquifer sediments at endogenous substrate concentrations when it was added at the beginning of the assay and significantly lowered the rate of subsequent N_2O production when it was added after N_2O production had begun (115 h) (Fig. 1). Total inhibition was also evident in similar assays using a continuous culture of denitrifying groundwater bacteria that was growing solely on the contaminated groundwater (Fig. 2), and chloramphenicol amendment in DEA-type assays with carbon-starved *P. denitrificans* resulted in at least a 50% reduction in enzyme activity relative to that of controls (Fig. 3A and 4A). New protein synthesis is not a reasonable explanation for the differences in measured denitrification activity between controls and chloramphenicol-amended flasks in this study, though a lag phase was evident with the sediment-slurry incubations (Fig. 1). A lag has often been observed with several types of activity measurements (C_2H_2 block, NO_3^- uptake, H_2 uptake, and thymidine uptake) with sediments and groundwater from the Cape Cod study site (10, 26, 27). We hypothesize that this lag results from the fact that bacteria in a groundwater or sediment sample require time to recover from the physical stresses imposed by groundwater sampling techniques. Incubations for which the results are depicted in Fig. 1 and 2 were performed at electron donor and acceptor levels that existed in the aquifer and chemostat; therefore, there were no increases in substrate concentrations to induce new denitrification enzyme synthesis. Sediments used in this study were collected from a zone in the aquifer in which O_2 concentrations were 5 μM or less (28), a value that is below concentrations reported to be inhibitory for denitrification in eutrophic lake sediments (18) or in the water column of the Baltic Sea (22). Dissolved oxygen was not present in the chemostat or pure culture samples. Significant O_2 repression of denitrification enzymes, prior to samples being introduced into incubation flasks, should not have been in effect for any of the samples used in this study. Therefore, subsequent derepression of denitrification enzyme synthesis initiated by anaerobiosis in the incubation flasks was unlikely. Most importantly, the addition of chloramphenicol to flasks that contained aquifer sediment and were producing N_2O, resulted in a significant decrease in the rate of N_2O production. We can only conclude that chloramphenicol amendment inhibited existing denitrification activity in all of the sample types which we examined in this study. Total or partial inhibition of existing DEA by chloramphenicol is not consistent with current understanding of this compound's utility in microbial activity assays. Chloramphenicol is classified as a bacteriostatic agent (21), and it is commonly assumed that addition of this compound should not affect the activity of existing enzymes.

The concentration of chloramphenicol used in this study (300 mg/liter) was chosen on the basis of work by others and titration experiments which we performed with a pure culture of *P. denitrificans*. MICs with respect to protein synthesis and growth range from 0.1 to 16 mg/liter for 6 species of gram-positive bacteria and 10 species of gram-negative bacteria (12). In practice, researchers performing DEA assays with environmental samples containing sediment have reported using concentrations ranging from 100 mg/liter (20, 23, 24) to as much as 5,000 mg/liter (an amount equaling twice the aqueous solubility of the compound [6]). These concentrations are significantly higher than the MICs because of concerns over sorption to sediment surfaces resulting in decreased antibiotic activity in incubation flasks. The recommended protocol for DEA assays with soils calls for 1,000 mg/liter (30). In experiments designed to gauge the MIC for preventing growth of *P. denitrificans* under the assay conditions we anticipated using at the Cape Cod study site, we found that concentrations greater than 200 mg/liter were required to prevent growth during a 48-h time course (3). The concentration of chloramphenicol which inhibited existing DEA in this study (300 mg/liter) is in the lower half of the range of concentrations that have been used and are recommended for DEA assays.

Smith and Tiedje (25) provided the basis for use of chloramphenicol in DEA assays with soils. In their paper, they described two distinct phases of denitrification that occurred in soils following imposition of anaerobiosis. Chloramphenicol did not decrease the rate of denitrification during the first phase, but did during the second. They interpreted this result to mean that the phase II activity was due to derepression of denitrification enzyme synthesis following 1 to 3 h of anaerobiosis. Firestone and Tiedje (6) examined temporal changes in the relative proportions of N_2O and N_2 produced by soils following imposition of anaerobiosis. In their study, chloramphenicol addition also lowered denitrification rates relative to those of control incubations, and a similar conclusion with regard to denitrification enzyme synthesis was reached. It is conceivable that the differences between chloramphenicol and nonchloramphenicol incubations in these two studies was caused by the same inhibition that we observed in the present study. Comparison of chloramphenicol and nonchloramphenicol rates has not been reported in subsequent studies employing DEA assays with soils (9, 15, 19, 20, 23, 24); therefore, it is unknown whether other researchers have encountered an effect similar to the inhibitory one we report here.

There are notable differences in the environmental conditions experienced by denitrifying populations from soil and the populations that experienced chloramphenicol inhibition of existing DEA in this study. Denitrification in soil can be limited by a lack of organic carbon or a suitable nitrogen oxide but is most frequently limited by the presence of O_2. Following wetting of soil, denitrifying bacteria experience periods of anaerobiosis during which denitrification enzymes become active and new enzyme synthesis may occur (5). These periods of activity are interspersed with periods during which the soil is aerobic and denitrification does not occur. This cyclical pattern of activity is in contrast to that for the groundwater environment at the Cape Cod site, where environmental conditions are relatively stable. Denitrification there is limited by a constant low supply of organic carbon (27), a fraction of which is unavailable for heterotrophic activity (2). Electron donor limitation is reflected by low total microbial biomass (11, 17) and community adenylate energy charge values indicative of metabolic stress (4). In the Cape Cod contaminant plume, most zones in which denitrification is occurring are not NO_3^- limited. Also, dissolved O_2 is very low or absent and not subject to the transient large increases that characterize the soil environment. Carbon limitation and the lack of NO_3^- limitation and O_2 inhibition were also the case for the *P. denitrificans* culture used for DEA assays, yet chloramphenicol addition to DEA assays of samples of that culture resulted in partial, rather than complete, inhibition of denitrification activity. This inhibition was observed even on day 0 of starvation, immediately after the culture had been harvested from exponential growth. Also noteworthy is the fact that the extent of inhibition changed during the 2-week starvation period (Fig. 3A and 4A). Interpretation of DEA assay results from a study in which environmental conditions varied significantly, either temporally or spatially or both, could be

troublesome if the various degrees of inhibition of DEA by chloramphenicol that we observed were in effect.

In this study, the inactivation that occurred cannot be attributed to chloramphenicol acting specifically on denitrification enzymes. Inactivation of other catabolic components could have resulted in the same outward effect, and further work will be necessary to examine, and possibly identify, a mechanism for this effect. Nevertheless, DEA was significantly decreased when chloramphenicol was present, and this decreased activity did not result from prevention of new enzyme synthesis. This result prevented our using a modified DEA assay at the Cape Cod study site and has obvious implications for the use of DEA assays in environments which are similar to that of the Cape Cod site. In such cases, parallel short-term control incubations without chloramphenicol could reveal whether the type of inhibition observed in this study was occurring. Although it has not been reported, it is conceivable that partial inhibition of DEA could also occur in DEA assays using soils. If this partial inhibition happens, interpretation of variations in DEA that occurred with time or between different environments could be in error. Finally, because the exact cause of this inhibition was not determined, an inhibitory effect on catabolic components other than denitrification enzymes cannot be ruled out. Until this effect is explained, caution in the design and interpretation of microbial activity assays that employ chloramphenicol to prevent new enzyme synthesis is recommended.

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