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Contributions of Autotrophic and Heterotrophic Nitrifiers to Soil NO and N_2O Emissions†

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Soil emission of gaseous N oxides during nitrification of ammonium represents loss of an available plant nutrient and has an important impact on the chemistry of the atmosphere. We used selective inhibitors and a glucose amendment in a factorial design to determine the relative contributions of autotrophic ammonium oxidizers, autotrophic nitrite oxidizers, and heterotrophic nitrifiers to nitric oxide (NO) and nitrous oxide (N_2O) emissions from aerobically incubated soil following the addition of 160 mg of N as ammonium sulfate kg^{-1}. Without added C, peak NO emissions of 4 μg of N kg^{-1} h^{-1} were increased to 15 μg of N kg^{-1} h^{-1} by the addition of sodium chlorate, a nitrite oxidation inhibitor, but were reduced to 0.01 μg of N kg^{-1} h^{-1} in the presence of nitrapyrin [2-chloro-6-(trichloromethyl)-pyridine], an inhibitor of autotrophic ammonium oxidation. Carbon-amended soils had somewhat higher NO emission rates from these three treatments (6, 18, and 0.1 μg of N kg^{-1} h^{-1} after treatment with glucose, sodium chlorate, or nitrapyrin, respectively) until the glucose was exhausted but lower rates during the remainder of the incubation. Nitrous oxide emission levels exhibited trends similar to those observed for NO but were about 20 times lower. Periodic soil chemical analyses showed no increase in the nitrate concentration of soil treated with sodium chlorate until after the period of peak NO and N_2O emissions; the nitrate concentration of soil treated with nitrapyrin remained unchanged throughout the incubation. These results suggest that chemoautotrophic ammonium-oxidizing bacteria are the predominant source of NO and N_2O produced during nitrification in soil.

Nitric oxide (NO) and nitrous oxide (N_2O) are two important gas species that affect the photochemistry and chemistry of the atmosphere of the earth. Both NO and N_2O are intimately involved in controlling the concentration of atmospheric ozone. In the troposphere, NO is the more active species and is involved in reactions that regulate both consumption and regeneration of ozone. High levels of tropospheric ozone cause eye watering and respiratory distress in humans and increase respiration in the leaves of plants, which depletes their carbohydrate supply (33). Nitric oxide is removed from the atmosphere by oxidation first to nitrogen dioxide (NO_2) and then to nitric and nitrous acids, which are the most rapidly increasing components of acid precipitation (19).

Nitrous oxide is chemically inert in the troposphere but readily diffuses to the stratosphere, where it becomes involved in a series of photochemical reactions that cause the destruction of ozone. Stratospheric ozone protects living organisms from solar UV light, which can cause skin cancer and has harmful effects on microbial life (1, 14). Nitrous oxide is also one of the atmospheric trace gases that have been implicated in global climate warming (16, 34, 42). In addition to the atmospheric interactions of these gases, NO and N_2O emissions represent a mechanism of soil N loss that creates imbalance in soil N budgets.

Biogenic production in soil is the principal source of atmospheric N_2O and may be a significant source of NO. Other sources include fossil fuel combustion, biomass burning, and lightning. The fractions of global NO_x (NO plus NO_2) emissions due to various sources are currently estimated to be as follows: fossil fuel combustion, 30 to 50%; biomass burning, 20 to 30%; lightning, 10%; and biogenic emissions from soil, 5 to 50% (29). Estimates of the magnitude of the global biogenic source range from 0 to 20 Tg of N $year^{-1}$ for NO and up to 10.4 Tg of N $year^{-1}$ for N_2O (3, 30). Greater uncertainty concerning biogenic NO production exists because N_2O has often been the only gaseous N oxide measured among the products of N transformation processes in soil. Recent evidence that NO emissions were several times larger than N_2O emissions from well-aerated soil in both natural and agricultural ecosystems (G. L. Hutchinson, W. D. Guenzi, and A. C. Tortoso, Abstr. 2nd Int. Symp. Biosphere-Atmosphere Exchange, 1986, p. 36) emphasizes the need to better characterize soil emissions of NO as well.

Nitrification, the biological oxidation of ammonium to nitrite or nitrate, is associated with the metabolism of certain chemoautotrophic bacteria, as well as with several species of heterotrophic microorganisms. Heterotrophs such as *Aspergillus flavus* and *Alcaligenes* spp. were reported to form nitrite in pure culture (13, 36), and Stroo et al. (40) presented evidence that heterotrophic organisms bring about nitrate formation in an acidic forest soil. Nevertheless, most nitrification in soil is accomplished by members of two genera of autotrophic bacteria: *Nitrosomonas* spp., which oxidize ammonium to nitrite, and *Nitrobacter* spp., which convert nitrite to nitrate (27). Several studies have demonstrated that in pure culture *Nitrosomonas europaea* has the capacity to produce NO and N_2O from ammonium or hydroxylamine (2, 21, 28, 44). In addition, field and laboratory studies have shown nitrification to be an important source of NO and N_2O in soil (8, 15, 24, 39).

Studies that use nitrification inhibitors such as acetylene or nitrapyrin [2-chloro-6-(trichloromethyl)-pyridine] have demonstrated that N_2O is produced as a direct result of chemoautotrophic nitrification (6, 8), but no similar attempt has been made to determine which group of nitrifiers is

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responsible for NO emissions from soil. Making that determination was the principal objective of this study. We hypothesized that chemoautotrophic nitrification is the predominant source of NO and, more specifically, that the organisms involved in the first step of this process—oxidation of ammonium to nitrite—are also responsible for formation of both NO and N_2O in aerobic soils.

MATERIALS AND METHODS

To test this hypothesis, we performed a 72-h aerobic soil incubation study that incorporated two nitrification inhibitors and a glucose amendment in a complete factorial design that included two levels each of the three factors and three replicates per treatment. The experiment was conducted with the flowthrough soil incubation system described by Hutchinson and Andre (24). Treated soil (depth, 1 cm) was placed in the bottom of each incubation jar and in the six sample vials suspended therein. The former served as a continuous, undisturbed source of the measured gases, while the latter provided convenient, preweighed samples for periodic chemical or microbiological analysis. Soil NO and CO_2 emission rates were determined by monitoring the concentrations of these gases in the humidified air that flowed continuously through each incubation jar. Exhaust air was also analyzed for N_2O by gas chromatography after 3, 6, 12, 24, 48, and 72 h of incubation.

Soil treatment. The soil was taken from an experimental site under no-till management at the High Plains Agricultural Laboratory near Sidney, Nebr. A winter wheat-fallow cropping rotation is practiced on the plots; N fertilizer is applied at the time of planting. Several samples taken from a depth of 0 to 10 cm were combined in plastic bags, packed on ice for transport to the laboratory, partially air dried to permit passage through a 2-mm sieve, and then stored in the refrigerator at 4°C.

To determine the relative contributions of various microbial groups to soil NO and N_2O emissions, we used nitrapyrin to block the autotrophic oxidation of ammonium to nitrite, sodium chlorate to block the autotrophic oxidation of nitrite to nitrate, and glucose to stimulate the activity of heterotrophic nitrifiers. Appropriate concentrations of the two inhibitors were determined in a series of preliminary experiments in which we monitored the response of the NO and CO_2 emission rates of the soil to a range of concentrations of each chemical (A. C. Tortoso, M.S. thesis, Colorado State University, Fort Collins, 1988). We were able to achieve complete inhibition of ammonium oxidizers but only partial inhibition of nitrite oxidizers without influencing the activity of nontarget microbial groups. The amount of added glucose was chosen to provide a ca. 12-h supply of readily available energy material to heterotrophic soil bacteria and fungi. Ammonium sulfate was added to all treatments to serve as substrate for nitrifying organisms; the amount was chosen so that the C/N ratio of added substrate was equal to 5 in glucose-amended soil. Dry treatment chemicals were added to the soil by using talcum as a carrier, as described by Anderson and Domsch (4). For inhibitors, the talcum/soil ratio was 0.5 g of talcum to 106.1 g of soil, and for substrates, it was 0.125 g of talcum to 106.1 g of soil. The talcum-chemical mixtures were prepared with a mortar and pestle.

To begin the experiment, the 166.1 g (dry weight) of soil incubated in each jar was wetted to -100 kPa water potential (31.4% water) by misting the soil with distilled water from a hand-operated pump spray bottle while mixing the soil and water on a large platform balance covered with waxed brown paper. Nitrapyrin (30 mg kg^{-1}), sodium chlorate (33 mg kg^{-1}), or both were added, and the soil was incubated at 25°C for 24 h to allow time for the inhibitors to dissolve and become uniformly distributed. Following preincubation, glucose (800 mg of C kg^{-1}), ammonium sulfate (160 mg of N kg^{-1}), or both were added and thoroughly mixed into the soil. Then 10 g (dry weight) of treated soil was added to each of six small sample vials, and the remainder was spread evenly across the bottom of the jar. After all vials were in place, the jars were sealed and gas analyses were initiated (24). One vial from each jar was extracted immediately; other sets of vials were removed after 6, 12, 24, and 48 h of incubation and were also extracted for chemical analysis. The sixth vial was removed from treatments without glucose after 6 h and used for ammonium and nitrite oxidizer population estimates. For treatments with glucose, the sixth vial was removed after 12 h and used to count heterotrophic microorganisms. After 72 h, soil samples were removed from the bottom of each incubation jar for chemical analyses, pH measurement, and ammonium and nitrite oxidizer population estimates.

Soil analyses. Inorganic soil N was extracted by shaking the soil with 1 N KCl (1:5 soil-to-solution ratio) on a wrist action shaker for 1 h. The suspensions were filtered through glass fiber filters and stored at 4°C. The concentrations of nitrite and nitrate in the extracts were determined by continuous-flow analyses (method no. 818-87T; Technicon Industrial Systems, Bran and Luebbe Analyzing Technologies, Elmsford, N.Y.). Soil pH was determined by a procedure based on the method described by McLean (32).

Microbiological analyses. Soil (10 g [dry weight]) was added to 95 ml of sterile solution and mixed for 2 min in a blender at high speed. From this, 10-fold serial dilutions were made. For heterotrophic counts, milk dilution bottles containing sterile 0.85% NaCl were used. Bottles containing sterile water were used for ammonium oxidizer counts, and bottles containing 1 mM phosphate buffer were used for nitrite oxidizer counts.

Enumeration of the ammonium and nitrite oxidizer populations was achieved by the most-probable-number method described by Schmidt and Belser (37). The medium of Sarathchandra (35) was used for ammonium oxidizers. Samples were observed and tested after 4 weeks; they were then tested weekly for 9 weeks for ammonium oxidizers and for 11 weeks for nitrite oxidizers.

Fungal, actinomycetous, and bacterial populations in the soil samples were determined by the plate count method with media described by Wollum (43). Martin rose bengal medium was used to enumerate fungal populations, actinomycetes were grown on starch-casein agar, and total bacterial counts were made with soil extract agar modified by the addition of 0.25 g of potassium monohydrogen phosphate.

Statistical analyses. Analysis of variance and regression analyses were performed by using GB-STAT Professional Statistics, Version 1.0 (Dynamic Microsystems, Inc., Silver Spring, Md.). Significance was assumed when P was ≤ 0.05.

RESULTS

Nitric oxide emissions during the 72-h incubation period, with and without added glucose, are presented graphically in Fig. 1A and B. Nitrous oxide emissions are presented in the same format in Fig. 2A and B. Evolution of both gases from soil treated with nitrapyrin was near zero and was significantly lower than that from the control soil, regardless of whether glucose or sodium chlorate or both were also added.

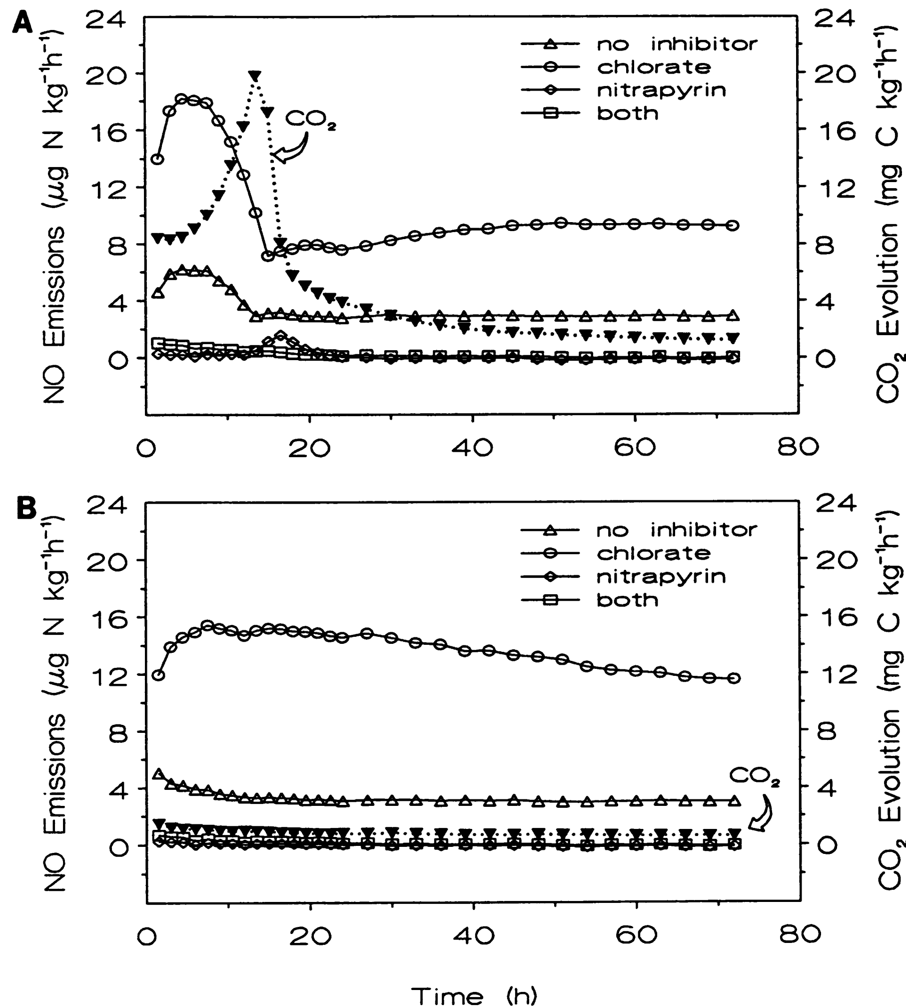

FIG. 1. Nitric oxide and carbon dioxide emission rates in soil treated with various inhibitors and amended (A) or not amended (B) with glucose. Datum points represent the means of three replications for NO and the means of all inhibitor treatments (n = 12) for CO_2.

The brief burst of NO and N_2O observed between 12 and 18 h from glucose-amended soil treated only with nitrapyrin was due entirely to the emission of these gases from one of the three replicates of that treatment. The burst probably resulted from denitrification that occurred in response to the spillage of water from the water reservoir of the jar during removal of the 12-h soil sample (for details of jar construction, see reference 24). As the added water was redistributed to drier soil around the spill area, the saturated soil again became aerobic and denitrification ceased.

The addition of sodium chlorate without nitrapyrin caused significantly higher NO and N_2O emission rates than did any other inhibitor treatment. Without glucose, NO evolution from this treatment rose to 15.4 μg of N kg^{-1} h^{-1} after 7.5 h and then steadily decreased over the remainder of the incubation period to 11.6 μg of N kg^{-1} h^{-1} (Fig. 1B). Nitrous oxide evolution from this treatment was much lower but followed a similar pattern, peaking early at 0.6 μg of N kg^{-1} h^{-1} and then declining slowly to about 0.4 μg of N kg^{-1} h^{-1} (Fig. 2B). Soil treated with glucose as well as sodium chlorate had even higher emission rates of both NO and N_2O. In this case, NO evolution peaked at 18.2 μg of N kg^{-1} h^{-1} at 4.5 h, dropped sharply to 7.2 μg of N kg^{-1} h^{-1} at 15 h, and then slowly increased to 9.4 μg of N kg^{-1} h^{-1} by the end of the incubation period (Fig. 1A). Again, N_2O evolution was much lower than that of NO but followed a similar pattern, peaking early at 1.1 μg of N kg^{-1} h^{-1} before declining sharply to 0.4 μg of N kg^{-1} h^{-1} as the supply of glucose was exhausted and then rising slowly to about 0.5 μg of N kg^{-1} h^{-1} by the end of the incubation (Fig. 2A).

After a brief lag phase, CO_2 evolution from glucose-amended soil increased steadily up to 14 h of incubation, when the supply of glucose was exhausted (Fig. 1A). At that time, the evolution rate began a rapid decline that slowed substantially after 18 h but continued throughout the remainder of the incubation. After 72 h, CO_2 evolution averaged 1.3 mg of C kg^{-1} h^{-1}, still about double the rate from soil not treated with glucose. Statistical analyses of CO_2 production rates during the exponential growth phase showed no differences due to treatments, indicating that the inhibitors had no effect on the growth of nontarget heterotrophic microorganisms. In the absence of added glucose, CO_2 evolution, which initially averaged about 1.4 mg of C kg^{-1} h^{-1}, exhibited a steady decrease to about one-half that rate after 72 h of incubation (Fig. 1B).

In the absence of nitrification inhibitors, nitrate concentration of the soil samples increased significantly (mean increase, 14 μg of N g^{-1}) during 72 h of incubation, indicat-

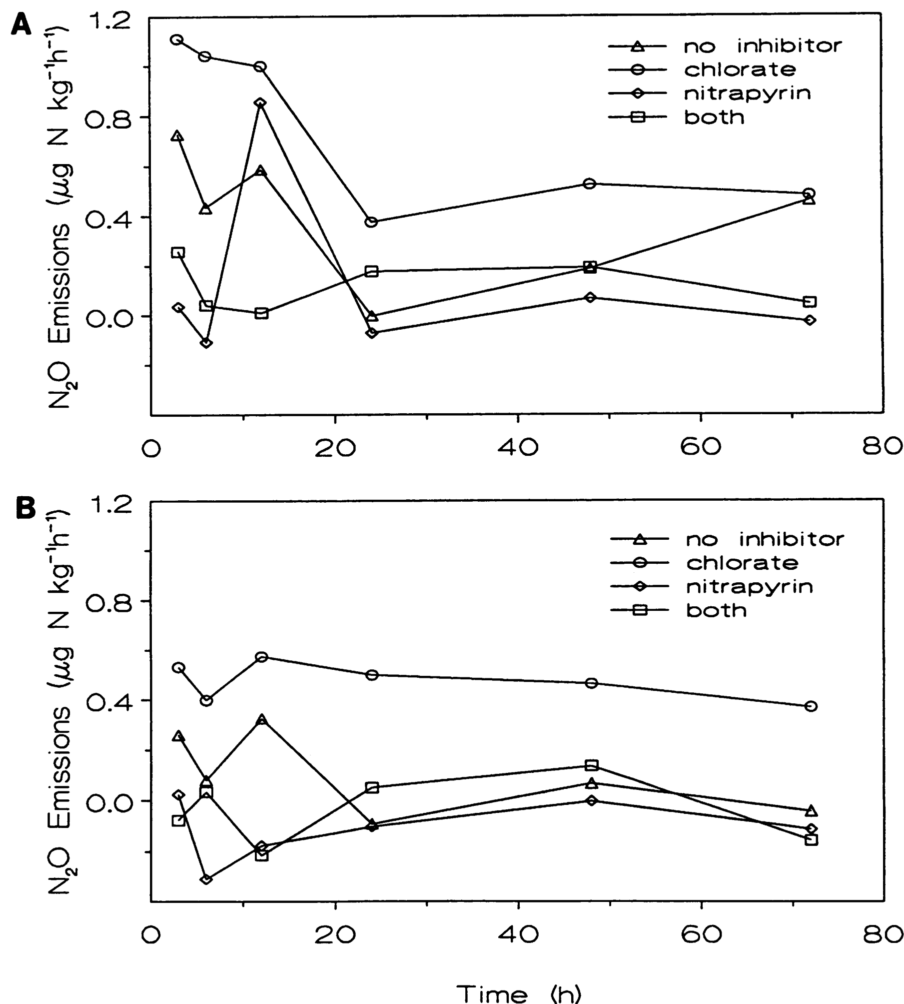

FIG. 2. Nitrous oxide emission rates in soil treated with various inhibitors and amended (A) or not amended (B) with glucose. Datum points represent the means of three replications.

ing that the soil contained an active population of nitrifying microorganisms (Fig. 3). The lack of change in the nitrate concentrations of samples treated with nitrapyrin confirms the effectiveness of this chemical as an inhibitor of ammonium oxidation. The nitrate concentrations of soils treated only with sodium chlorate did not change during the first 24 h of incubation, but significant increases were observed during the remainder of the incubation. Apparently, the inhibition of nitrite oxidation by this chemical was incomplete at the concentration we used. The addition of glucose had no significant effect on nitrate accumulation.

Neither nitrapyrin nor sodium chlorate significantly influenced total actinomycetous, fungal, or bacterial populations sampled 12 h after soil amendment with glucose (Table 1). The inhibitors also had no significant influence on the population of ammonium-oxidizing bacteria, and most-probable-number estimates of the populations of these organisms were the same after 6 and 72 h of incubation (Table 2). Results for nitrite-oxidizing bacteria were similar, except that in soil treated with sodium chlorate the population of this group was significantly reduced at 72 h.

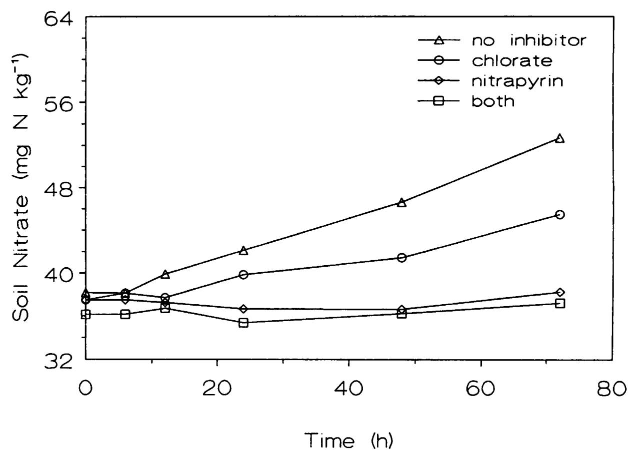

FIG. 3. Nitrate concentrations in soil treated with various inhibitors. Datum points represent the means of three replications for soils with and without a glucose amendment (n = 6).

TABLE 1. Populations of heterotrophic microbial groups after 12-h incubation of glucose-amended soil treated with various inhibitors

Treatment	CFU[a] g of soil^{-1}		
	Bacteria (10^6)	Actinomycetes (10^6)	Fungi (10^3)
No inhibitors	49 ± 20	51 ± 2	136 ± 66
Chlorate	65 ± 13	54 ± 16	70 ± 27
Nitrapyrin	53 ± 16	46 ± 19	71 ± 31
Chlorate + nitrapyrin	60 ± 8	50 ± 12	115 ± 59

[a] Values are means ± standard errors (n = 3).

DISCUSSION

Nitric and nitrous oxide emission rates of the control soil, which averaged 4.0 and 0.1 μg of N kg^{-1} h^{-1}, respectively, were similar to those reported for other laboratory studies. For example, Johansson and Galbally (25) reported NO emissions from aerobic soil columns of 3.6 μg of N kg^{-1} h^{-1}, and Blackmer et al. (8) measured an N_2O production of 0.1 μg of N kg^{-1} h^{-1} from soil amended with ammonium sulfate (200 mg of N kg^{-1}). Freney et al. (18) found that N_2O emissions averaged 0.16 μg of N kg^{-1} h^{-1} from soil at a water potential similar to that we used, but they measured higher levels of emission from wetter soils. Other researchers also reported that N_2O emissions were enhanced by increasing soil water content (20, 26) and that N_2O production is more strongly influenced by oxygen concentration than is NO production (2). The large ratio of soil NO/N_2O emissions observed in this study is similar to that reported by McKenney et al. (31) and by Hutchinson et al. (G. L. Hutchinson, A. C. Tortoso, and W. D. Guenzi, Agron. Abstr., 1987, p. 185).

In all treatments without nitrapyrin, initial NO and N_2O emission rates were elevated by the addition of glucose (cf. Fig. 1 and 2) but began declining as heterotrophic microorganisms began their exponential growth response to the added C. Emission levels of these two gases reached minimums coincident with exhaustion of the added glucose and then climbed slowly during the remainder of the incubation, but the emissions never recovered to the levels exhibited by soil not treated with glucose. The initial enhancement of N oxide emissions probably resulted from a slight decrease in the oxidation-reduction potential of the soil caused by the increase in competition for oxygen associated with heterotrophic metabolism of the added glucose. With the onset of the exponential growth phase, however, this enhancement was overcome by the immobilization of ammonium in microbial biomass. We estimated that approximately one-half of the added ammonium would be immobilized during glucose oxidation, a reduction similar to the observed 60% reduction in N oxide emissions over this period. Subsequent incubation experiments confirmed that the aerobic NO and N_2O emission rates of the soil are directly related to the ammonium concentration of the soil.

Soil samples treated with nitrapyrin exhibited no significant changes in nitrate concentrations during incubation and no significant differences in CO_2 production or numbers of chemoautotrophic or heterotrophic microorganisms from those measured in the control soil. Apparently, at the concentration we used (30 mg kg^{-1}), nitrapyrin completely inhibited ammonium oxidation without affecting the activity of other microbial groups. Other studies have also shown nitrapyrin to be a potent inhibitor of the nitrification process in soil (11, 22, 23) and to be specific for chemoautotrophic ammonium oxidizers (12, 38). Because inhibition of ammonium oxidation by nitrapyrin also suppressed soil NO and N_2O emission levels to near zero, we concluded that in aerobic soils both gases either were direct metabolic products of chemoautotrophic ammonium-oxidizing bacteria or resulted from other soil processes dependent on these organisms as a source of nitrite. Similar results have been observed for N_2O emissions in other studies that used nitrapyrin as a nitrification inhibitor. For example, Blackmer and Bremner (7) found that soil N_2O emissions were greatly reduced by the addition of nitrapyrin and concluded that most of the N_2O evolved from ammonium-treated soil was generated by chemoautotrophic, nitrifying bacteria. Aulakh et al. (6) also observed that nitrapyrin stopped the oxidation of ammonium and reduced the evolution of N_2O.

Among the potential fates of nitrite in aerobic soil, the most common is oxidation to nitrate by chemoautotrophic bacteria, a process purported to be specifically inhibited by sodium chlorate (27). The inhibitory effect of this chemical on nitrite oxidation in our study was evidenced by increased soil nitrite concentration (data not shown), lack of a significant increase in nitrate concentration during the period of peak NO and N_2O emissions, and a significant reduction in the population of chemoautotrophic nitrite-oxidizing bacteria after 72 h. Because the addition of sodium chlorate without nitrapyrin obviously repressed nitrite oxidation yet resulted in significantly higher NO and N_2O emission levels, we concluded that chemoautotrophic nitrite-oxiding bacteria were not directly responsible for the production of these two gases.

A second process by which transformations of soil nitrite might result in production of NO and N_2O is heterotrophic nitrite oxidation, which we evaluated by amending the test soil with glucose to stimulate heterotrophic microbial growth. This substrate was chosen because most known soil bacteria and fungi can use it as a source of both energy and

TABLE 2. Most probable numbers of ammonium and nitrite oxidizers after 6- or 72-h incubation of soil treated with various inhibitors but not amended with glucose

Treatment	No. of cells[a] g of soil^{-1}			
	Ammonium oxidizers after:		Nitrite oxidizers after:	
	6 h	72 h	6 h	72 h
No inhibitors	2.8×10^4 (0.85–9.2)	13.0×10^4 (3.9–42.9)	2.3×10^6 (0.70–7.6)	3.3×10^6 (1.0–10.9)
Chlorate	1.7×10^4 (0.52–5.6)	3.1×10^4 (0.9–10.2)	2.3×10^6 (0.70–7.6)	7.9×10^4[b] (2.4–26.1)
Nitrapyrin	1.1×10^4 (0.33–3.6)	3.3×10^4 (1.0–10.9)	1.7×10^6 (0.50–5.6)	3.3×10^6 (1.0–10.9)
Chlorate + nitrapyrin	4.6×10^4 (1.4–15.2)	2.3×10^4 (0.7–7.6)	2.3×10^6 (0.70–7.6)	4.9×10^4[b] (1.5–16.2)

[a] Values in parentheses represent 95% confidence intervals.
[b] Values are significantly different from other values in this column on the basis of 95% confidence intervals.

C for cell synthesis (5). The importance of appropriate C and N substrates in media used to examine heterotrophic nitrification was discussed by Wallace and Nicholas (41) and Focht and Verstraete (17). Although the addition of glucose enhanced NO and N_2O emissions during the first few hours of the incubation, the emission rates peaked and began declining at about the same time that exponential growth of heterotrophic microorganisms was commencing. By the time heterotrophic activity reached a maximum (as measured by CO_2 evolution), NO emissions had decreased to a level lower than that from soil not treated with glucose. We interpreted the negative correlation between N oxide emission levels and the activity of heterotrophic microorganisms as evidence that this group could not have been responsible for more than a very small fraction of NO and N_2O production in the experimental soil.

Chemodenitrification is an abiological process for soil nitrite destruction that also yields NO and N_2O. Previous studies of this process have shown that disproportionation of nitrous acid is most significant in acid soils with a pH of less than 5 (39), especially those with high organic-matter content (9). Solution studies by Bremner and Nelson (10) confirmed that self-decomposition of nitrous acid is negligible at a pH of 6. The organic C content of the soil used in our study was 2.05%, lower than any of the soils used by Blackmer and Cerrato (9), and the pH after 72 h of incubation ranged from 5.94 to 6.39. Therefore, we concluded that the fraction of observed NO and N_2O emission levels resulting from chemodenitrification was probably very small compared with that arising from biologically mediated nitrification processes in the test soil. Additional support for this conclusion was obtained in earlier studies (A. C. Tortoso, G. L. Hutchinson, and W. D. Guenzi, Agron. Abstr., 1986, p. 190–191), which demonstrated that sterilization of this soil completely eliminated N oxide emissions.

Because the three known aerobic processes involving transformation of soil nitrite did not appear to be actively involved in production of the NO and N_2O measured in this study, we concluded that both gases were direct metabolic products of chemoautotrophic ammonium-oxidizing bacteria. The amounts of NO and N_2O produced by these organisms amounted to 1.7 and 0.02%, respectively, of the amount of N nitrified (as measured by nitrate accumulation). If it is assumed that these ratios are constant for all N that undergoes nitrification, then we estimate the resulting global flux of NO and N_2O to the atmosphere at 6 and <1 Tg of N year^{-1}, respectively. Similar estimates were obtained by extrapolating our observed emission rates to global amounts by multiplying by the ratio of soil areas exposed to the atmosphere. By comparison, the total biogenic NO_x flux was estimated by Logan (29) to be 4 to 16 Tg of N year^{-1} and the biogenic N_2O flux was estimated by McElroy and Wofsy (30) to be 10.4 Tg of N year^{-1}. Our results support the contentions that emission of NO, rather than N_2O, is the principal gaseous N loss mechanism during nitrification in soil (G. L. Hutchinson, A. C. Tortoso, and W. D. Guenzi, Agron. Abstr., 1988, p. 218) and that anaerobic soil processes, rather than nitrification, are the principal biogenic source of atmospheric N_2O (18, 20, 26).

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