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Specific Inhibition of Nitrite Oxidation by Chlorate and Its Use in Assessing Nitrification in Soils and Sediments

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A method was developed to determine the ammonium oxidation rate (potential) of unenriched natural samples by measuring the nitrite produced in shaken slurries. Addition of chlorate to the samples prevented nitrite from being oxidized to nitrate. The effectiveness and specificity of chlorate were tested with pure cultures of nitrite and ammonium oxidizers, as well as in soil and sediment slurries. It was concluded that chlorate had relatively little inhibitory effect on ammonium oxidation. However, under some conditions chlorate was not completely effective in blocking nitrite oxidation, and the causes of this were investigated. The technique was designed to check for incomplete blockage.

Normally, nitrification proceeds in two steps, with ammonium being oxidized to nitrite by one group of autotrophic nitrifiers and nitrite to nitrate by another. Since nitrite is being oxidized as it is being produced, the rate at which ammonium is oxidized is equal to that at which nitrite plus nitrate accumulates. It is clear that if nitrite oxidation could be completely and specifically blocked, the rate at which nitrite alone accumulates would equal the rate of ammonium oxidation. This would be an advantage where automated methods for nitrate plus nitrite are not available, since other methods for nitrate (or nitrite plus nitrate) are less convenient and possibly less sensitive than for nitrite alone.

Although Lees and Simpson (8) showed 20 years ago that chlorate is a specific inhibitor of nitrite-oxidizing bacteria, there has been relatively little subsequent work with this inhibitor. According to Lees and Simpson, low concentrations of chlorate (i.e., 10^{-5} M) inhibit autotrophic nitrite oxidizer growth, whereas it takes much higher concentrations (i.e., 10^{-3} to 10^{-2} M) to completely inhibit nitrite oxidation. Although some use has been made of low concentrations of chlorate to inhibit growth (6), none has been made of high concentrations of chlorate to inhibit nitrite oxidation. In this report the selectivity of chlorate inhibition on the oxidation of nitrite, with respect to possible inhibition of ammonium oxidation, is investigated. The aim was to develop a method for measuring ammonium oxidizer activity in which only nitrite need be measured.

MATERIALS AND METHODS

Sediments and soils. Initial work was done with intertidal sediments collected at Delaware Inlet, Nelson, New Zealand. Typical results with only one of these sediments, which is classified as a silty sand (7), are presented here. Two pasture soils were also used and are described as a Wakapuaka sandy loam and a Ronga silty loam (5). Both soils were in permanent clover grass pastures (grazed by either sheep [Wakapuaka] or cattle [Ronga]).

Pure cultures. Three *Nitrosomonas* strains (*N. europaea*, WH-2, and D4-1), one *Nitrosolobus* strain (Fargo), one *Nitrosospira* strain (AV), and one *Nitrobacter* strain (*N. winogradskyi*) were tested for sensitivity to chlorate. With the exception of *Nitrosomonas* D4-1, a marine *Nitrosomonas* strain isolated from Delaware Inlet by the method of Belser and Schmidt (2), all strains have been described previously (3).

The freshwater strains of ammonium and nitrite oxidizers were grown in media described previously (1, 2), and the marine *Nitrosomonas* strain was grown in ammonium oxidizer medium (2) supplemented with marine salts. The marine salts supplement was prepared as follows. Solution A contained 25 g of NaCl, and 10.6 g of $MgCl_2 \cdot 6H_2O$ per 100 ml of distilled water; solution B contained 5.0 g $CaCl_2 \cdot 2H_2O$ in 10 ml of water. Solutions A and B were sterilized separately (121°C, 15 min) and combined after cooling. One milliliter of this supplement was aseptically added to tubes containing 10 ml of sterile ammonium oxidizer medium.

Effect of chlorate on the growth and oxidation kinetics of nitrifiers in pure culture. For ammonium oxidizers, flasks containing 200 ml of growth medium were inoculated with 0.5 ml of exponentially growing cultures. Growth was allowed to proceed in these flasks until a 0.05 to 0.10 mM concentration of product had been produced. Aliquots (10 ml) were then transferred to sterile culture tubes. These tubes were incubated overnight before chlorate was added. Triplicate tubes received either 0.1 or 0.3 ml of 1 M chlorate or no addition (i.e., final concentrations of 0, 10, and 30 mM chlorate). Nitrite was analyzed at least daily during the incubation. Incubations at 23°C were continued for at least 3 days after addition of chlorate. Specific growth rates were calculated by using only nitrite accumulation data, according to the procedure of Belser and Schmidt (FEMS Microbiol. Lett., in press).

Nitrobacter growth studies were similar except that the final chlorate concentrations were 0, 3, and 10 mM. Nitrite disappearance was monitored.

Ammonium oxidizer activity measurements. Activity measurements were started within 2 h of collecting soil or sediment samples. For soils, 75 g of soil (moist) was sieved (2 mm) and blended (30 s, high-speed Waring blender) with 350 ml of ammonium phosphate buffer [1 mM potassium phosphate, pH 7.2; 0.5 mM $(NH_4)_2SO_4$]. Aliquots (50 ml) of the slurry were distributed in six 250-ml screw-cap bottles, and to four of these 0.5 ml of 1 M $NaClO_3$ was added. All the bottles were incubated with shaking at 25°C. Nitrite was measured periodically, and when a significant amount of nitrite had accumulated, 5 μl of 20% nitrapyrin [2-choro-6-(trichloromethyl) pyridine; Dow N-serve 93.7%, lot 37] in dimethyl sulfoxide was added to two of the bottles with chlorate and to two bottles without chlorate. To the two remaining bottles containing chlorate 5 μl of dimethyl sulfoxide alone was added. Nitrite was measured at timed intervals for the next 12 to 24 h. The same procedure was used for sediments, except that they were not sieved, and slurries were made with aged filtered seawater (200 g of sediment per 500 ml of aged seawater).

The effect of ammonium concentration on the oxidation kinetics in soil slurries was checked on several occasions by additions of 0, 0.5, or 2.5 μmol of $(NH_4)_2SO_4$ per ml of replicate slurries.

During one experiment nitrate and ammonium were measured. Since about 25 ml of supernatant is required for the nitrite-plus-nitrate analysis (10), the scale of the incubations was increased. A slurry was prepared as before with 350 g of soil added to 2,500 ml of buffer (1 mM potassium phosphate, pH 7.2). Twelve bottles (800 ml) containing 200 ml of slurry were prepared. There were four treatments done in triplicate: (i) no additions; (ii) 2 ml of 1 M chlorate; (iii) 20 μl of 20% nitrapyrin; (iv) both chlorate and nitrapyrin.

Effect of chlorate on the K_m and V_{max} of nitrite oxidation. Soil slurries were prepared by blending 200 g of soil (Ronga) with the phosphate buffer, resulting in a final ratio of moist soil to buffer of 1:5. A total of 50 ml of this slurry was added to each of 20 screw-cap bottles (250 ml). Four sets of four bottles each received different nitrite additions, with approximate final concentrations of 0.025, 0.050, 0.10, and 0.20 mM nitrite. Two bottles at each nitrite concentration received 0.5 ml of 1 M chlorate, and all 16 of these bottles received 5 μl of 20% nitrapyrin. Of the remaining four bottles without added nitrite, two received 0.5 ml of 1 M chlorate and the other two received no additions.

The disappearance of nitrite was measured in the 16 bottles containing nitrapyrin. The bottles without chlorate were sampled at 0.5-h intervals; those with chlorate were sampled at 1- to 2-h intervals initially and at 21 h (overnight). The K_m and V_{max} for nitrite oxidation were obtained by the method of Lineweaver and Burke. Accumulations of nitrite in the four bottles without nitrapyrin were measured periodically. Ammonium was measured periodically in eight of the bottles with nitrapyrin (four with chlorate [0.025 and 0.050 mM NO_2^-] and four without chlorate [0.025 and 0.050 mM NO_2^-]) and in all of the bottles without nitrapyrin. Ammonium accumulation in bottles with nitrapyrin gives a measure of the ammonification rate.

Sampling and chemical analysis. The bottles were sampled without interrupting shaking. Samples of 3 or 5 ml were normally taken (25 ml when nitrate analysis was done). For ammonium analysis samples were diluted 1:1 with 4 M KCl and were vigorously shaken for 15 min. Supernatant solutions were prepared by centrifuging (Beckman JA21B centrifuge, JA20 or JA21 head) at 12,000 × *g* for 10 min.

Nitrite was measured by a modified Griess-Illosvay technique (4), ammonium was measured by the method of Weatherburn (11), and nitrate plus nitrite was measured by a cadmium reduction technique (10). Results are expressed per gram of oven-dried soil (105°C) except where noted.

RESULTS

Initial studies with ammonium oxidizers were done with *N. europaea* with and without chlorate (10 mM). These results are given in Fig. 1. Seventeen hours after the addition of chlorate, the cultures with chlorate had produced an average nitrite concentration of 0.090 ± 0.002 mM, whereas the controls had produced an average of 0.091 ± 0.004 mM. Subsequently, the difference in concentrations became larger. This may have been caused by inhibition of growth rather than by a specific inhibition of the oxidation of ammonium.

To assess the effect of chlorate on the growth of ammonium oxidizers, specific growth rates were calculated, using only nitrite data, for *N. europaea* and the other ammonium oxidizers as

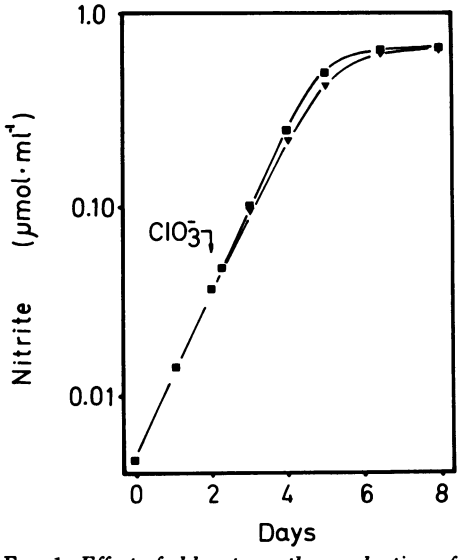

FIG. 1. *Effect of chlorate on the production of nitrite during exponential growth of N. europaea. Symbols: ■, without chlorate; ▼, nitrite after addition of 10 μmol of chlorate per ml.*

TABLE 1. *Specific growth rates for ammonium oxidizers as a function of chlorate concentration in the growth medium*

Cultures	Specific growth rate[a] (h^{-1})		
	Control	Chlorate	
		10 mM	30 mM
N. europaea	0.0362 ± 0.0002	0.0344 ± 0.0011	ND[b]
WH-2	0.0223 ± 0.0020	0.0213 ± 0.0016	0.0217 ± 0.0007
D41 (marine)	0.0255 ± 0.0027	ND	0.0244 ± 0.0011
Nitrosolobus Fargo	0.0290 ± 0.0007	0.0298 ± 0.0007	0.0309 ± 0.0017
Nitrosospira AV	0.0279 ± 0.0006	0.0266 ± 0.0024	0.0228 ± 0.0028

[a] Each value is the average of three incubations ± the standard deviation.
[b] ND, Not done.

functions of chlorate concentration (Table 1). This appears to be valid when cultures are growing exponentially (Belser and Schmidt, in press). The production of nitrite in individual tubes was highly correlated to the exponential growth equation. The worst correlation was with the WH-2 strain, being an average of 0.923 for all individual incubations. All other incubations had correlation coefficients above 0.980, with *N. europaea* and the *Nitrosospira* strain being above 0.998 in all incubations.

The effects of 3 and 10 mM chlorate on the exponential growth of *N. winogradskyi* are shown in Fig. 2. Complete and immediate inhibition of oxidation was obtained with 10 mM chlorate, whereas there was a lag before 3 mM chlorate completely inhibited nitrite oxidation.

The final procedure adopted for measuring ammonium oxidation rates in natural samples was to incubate replicate slurries in shaken bottles with and without chlorate present. Nitrite was allowed to accumulate with periodic sampling. Three to four samples were taken during a period of 7 to 24 h, and then nitrapyrin was added. Sampling and nitrite analysis were continued.

Figure 3 shows the results of such a slurry incubation with the intertidal sediment. It can be seen that nitrite accumulates at a constant rate in flasks with chlorate present. After nitrapyrin was added the concentration of nitrite remained constant with time. Since nitrapyrin is a specific inhibitor of autotrophic ammonium oxidation (9), this demonstrates not only that the nitrite was being produced solely from autotrophic ammonium oxidation, but also that the chlorate was effectively blocking nitrite oxidation. If the block had not been effective, nitrite would have decreased with time. In bottles without chlorate added nitrite accumulates at a slower rate, and when nitrapyrin was added nitrite decreased.

The results of incubations of two pasture soil

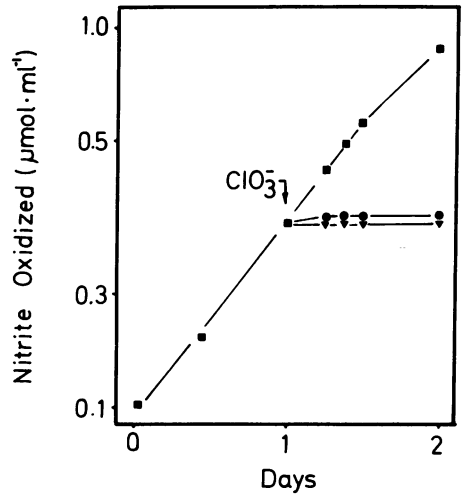

FIG. 2. *Effect of chlorate on the oxidation of nitrite during exponential growth of N. winogradskyi. Symbols: ■, without chlorate; ●, after addition of 3 μmol of chlorate per ml; ▼, after addition of 10 μmol of chlorate per ml.*

slurries are shown in Fig. 4. Chlorate appears to be an effective inhibitor of nitrite oxidation with the Wakapuaka soil, since the nitrite concentration remained constant after nitrapyrin addition. This was not the case for the Ronga soil.

Two sets of studies using the Ronga soil (experiments 1 and 2) were done on successive weeks to measure the effects of chlorate on the kinetics of nitrite oxidation (i.e., the effects on V_{max} and K_m; Table 2). On addition of chlorate, the apparent V_{max} decreased between 42 and 58%, whereas the K_m's increased between a factor of 7 and 10. It is clear that 10 mM chlorate does not completely block nitrite oxidation in soils. Since the K_m is the kinetic parameter most affected, the inhibition of the nitrite oxidizer activity will be a function of nitrite concentration. The amount of inhibition predicted from

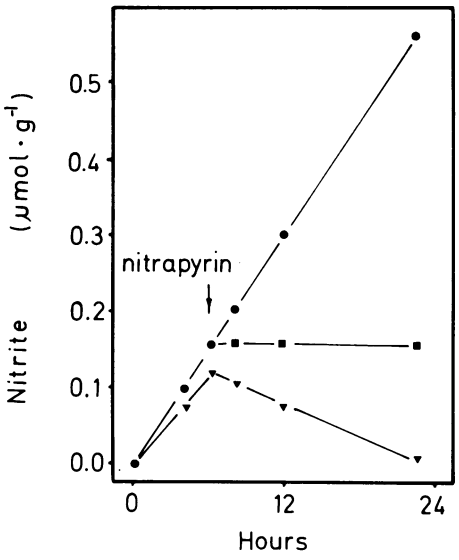

FIG. 3. *Nitrite production for slurry incubations of an intertidal sediment. Symbols: ●, chlorate present; ■, nitrite after nitrapyrin was added to slurries with chlorate present; ▼, no chlorate, with nitrapyrin added during incubation.*

the Michaelis-Menten equation at three nitrite concentrations is given in Table 3. It can be seen that at low nitrite concentrations the block is much more effective, being in the range of 93 to 94%.

Ammonium oxidation rates (i.e., accumulation of nitrite with chlorate present) were also measured during these two studies. These rates were 0.60 and 0.058 μmol g^{-1} h^{-1} for the first and second incubations. These are 60 to 70% maximum rates of nitrite oxidation during these studies. This percentage is slightly higher than observed in the initial incubations (Fig. 4), where the ammonium oxidation rate was about 50%.

Since ammonium was also analyzed during these studies, an independent measure of the ammonium oxidation rate was possible. The rate that ammonium concentrations changed in the slurries, however, is not the oxidation rate, since ammonification was occurring simultaneously. The rate that ammonium concentration (s) changed (ds/dt) is equal to the ammonification rate (A) minus the ammonium oxidation rate r ($r = [ds/dt] - A$). The ammonification rate was measured in bottles with nitrapyrin present ($r = 0$ and $A = [ds/dt]$). The values for the ammonium oxidation rates with and without chlorate present are given in Table 4 (columns 1 and 2) for experiments 1 and 2. Although these rates compare favorably with rates determined by nitrite accumulation (column 3), it appears that the rates with chlorate present may be less than those without chlorate.

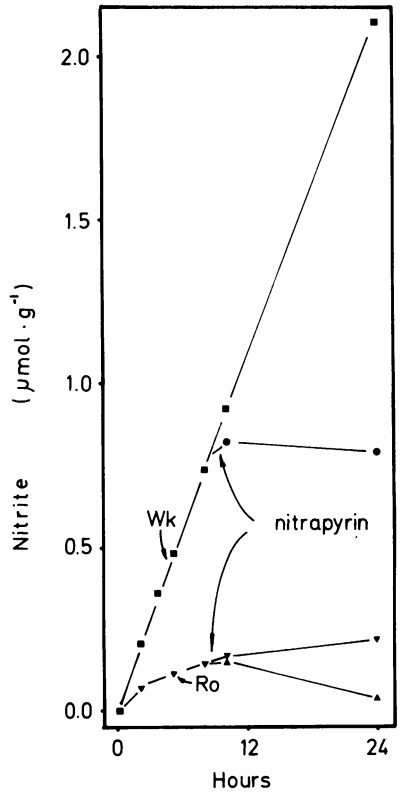

FIG. 4. *Nitrite production for slurry incubations of two pasture soils. Symbols for Wakapuaka soil: ■, nitrite production with chlorate; ●, nitrite after addition of nitrapyrin. Symbols for Ronga soil: ▼, nitrite production with chlorate; ▲, nitrite after addition of nitrapyrin. (Nitrite expressed in micromoles per gram [wet weight] of soil.)*

TABLE 2. *Effect of 10 mM chlorate on the oxidation of nitrite in soils*

Sample	V_{max} (μmol g^{-1} h^{-1})	K_m (mM)	r^a
1			
$-ClO_3^-$	0.0900	0.0303	0.938
$+ClO_3^-$	0.0375	0.209	0.973
2			
$-ClO_3^-$	0.1090	0.0423	0.997
$+ClO_3^-$	0.0635	0.468	0.968

[a] Correlation coefficient of the linear regression to a Lineweaver-Burke plot.

To check on the possibility that chlorate may have inhibited ammonium oxidation in the soil slurries and to determine the amount of leakage of nitrite past the chlorate block, nitrate was measured in a third group of incubations (experiment 3, Table 4). In this experiment triplicate incubations were done: (i) with chlorate (10

TABLE 3. *Percent inhibition of nitrite oxidation at various concentrations of nitrite in the presence of 10 mM chlorate*

Sample	% Inhibition at nitrite concn:		
	0.01 mM	0.05 mM	0.10 mM
1	92.3	87.1	82.4
2	93.7	89.6	85.4

TABLE 4. *Effect of chlorate on the oxidation of ammonium*

Expt	Ammonium oxidation rate (μmol g^{-1} h^{-1}) based on:				
	Changes of NH_4^+		Changes in NO_2^- (ClO_3^-)	Changes in NO_2^- + NO_3^-	
	Control	ClO_3^-		Control	ClO_3^-
1	0.089	0.083	0.085	ND[a]	ND
2	0.083	0.063	0.081	ND	ND
3	0.077	0.084	0.074	0.095	0.085

[a] ND, Not done.

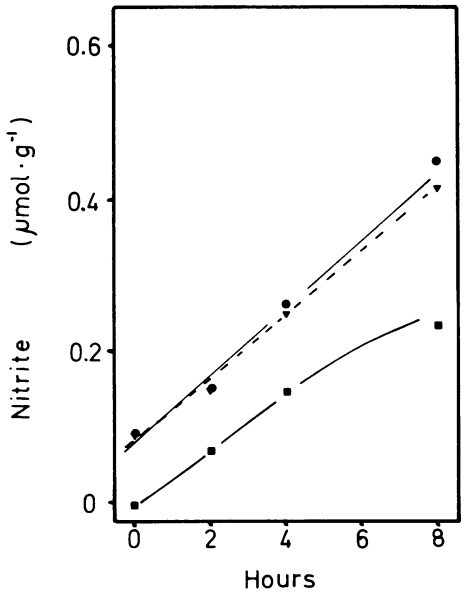

FIG. 5. *Comparison of the rates of ammonium oxidation determined by: ■, the accumulation of nitrite in the presence of chlorate; ▼, the accumulation of nitrite plus nitrate in the presence of chlorate; ●, the accumulation of nitrite plus nitrate without chlorate. (Nitrite expressed in micromoles per gram [wet weight] of soil.)*

mM); (ii) with nitrapyrin (20 μg/liter); (iii) with both chlorate and nitrapyrin; (iv) with no additions. Rates of ammonium oxidation based only on ammonium data were calculated as before. No difference was seen between the rates with and without chlorate present (Table 4, experiment 3, columns 1 and 2).

When oxidation rates were calculated on the basis of the accumulation of nitrite plus nitrate (see Fig. 5), the rate of ammonium oxidation without chlorate (Table 4, column 4) appeared to be higher than that with chlorate (column 5). Subsequent analysis of variance of the rates (slopes of the regression equation), however, were inconclusive ($0.3 > P > 0.1$), and it could not be concluded that the rates were significantly different.

The rate of nitrite accumulation is also given in Fig. 5. The initial rate of accumulation was 0.052 μmol g^{-1} h^{-1}, which was 86% of the rate based on nitrite plus nitrate (measured on the same bottles). This was in the range of leakage predicted in Table 3.

Experiment 3 also allowed an estimate of the effectiveness of the nitrapyrin block on ammonium oxidation. The rate that nitrite plus nitrate accumulated in the presence of nitrapyrin was 1.2 nmol g^{-1} h^{-1}, which was 2.5% of the rate of accumulation without nitrapyrin.

There were two features of the changes in ammonium concentrations that are as yet unexplained. First, in all incubations there was an initial burst of ammonium production during the first 3 to 6 h, after which the rate became constant for the next several days. Ammonification rates were determined during these constant rate periods. Second, ammonium concentrations were significantly higher in incubations with chlorate than in those without chlorate even though no ammonium was added.

It was also observed during the course of these studies that addition of ammonium did not increase the ammonium oxidation rate. Thus, the ammonium oxidation rates measured in experiments 1 through 3, where ammonium was not added, should not be any lower than in incubations where 1 mM ammonium was present (slurries had between 0.15 and 0.25 mM NH_4^+).

DISCUSSION

The procedure developed here has two advantages over methods where both nitrite and nitrate are measured. First, when automated methods are not available, nitrite is much simpler to measure and requires a smaller sample size. Second, in soils with high nitrate background, this method will be much more sensitive, since nitrite will normally be undetectable at the beginning of the incubation.

In order for this method to be most accurate in measuring the ammonium oxidation rate in a slurry, (i) chlorate must not inhibit ammonium oxidation and (ii) chlorate must completely block nitrite oxidation. If either or both of these

conditions do not exist, this method will underestimate the rate of ammonium oxidation.

Chlorate appears to have relatively little inhibitory effect on the growth of ammonium oxidizers. Of the studies in Table 1, where more than one concentration of chlorate was used, only the *Nitrosospira* strain shows a consistent negative correlation between chlorate concentration and specific growth rate ($r = -0.778$). From the slope of the regression line, one would expect about a 6% inhibition of growth at 10 mM chlorate with *Nitrosospira*. This is about the same as the apparent inhibition for *N. europaea* based on only one concentration of chlorate. The correlation coefficients between specific growth rate and chlorate concentration for *Nitrosomonas* strain WH-2 and the *Nitrosolobus* strain are −0.210 and 0.668, respectively.

The results of studies on the kinetics of chlorate inhibition (Fig. 5, Table 4) indicate that in slurries, inhibition of nitrite oxidation can be less than complete. This does not appear to present a problem when the V_{max} for nitrite oxidation is less than the rate of ammonium oxidation (e.g., sediment, Fig. 3; Wakapuaka soil, Fig. 4). However, when the V_{max} is larger (e.g., Ronga soil, Fig. 4), a significant underestimation can occur if the rate of nitrite oxidation is averaged over a prolonged incubation. Since chlorate appears to affect mainly the K_m of the reaction, the initial rate of the reaction is the best estimate of the ammonium oxidation rate. This is because leakage will be least at low nitrite concentrations. In many cases this initial rate will be a good estimate of the ammonium oxidation rate. Cases where leakage presents a problem are readily indicated by the decrease in nitrite after the addition of nitrapyrin.

Table 4 summarizes the three methods used here to estimate ammonium oxidation rates with the Ronga soil. The chlorate method gives good agreement with the other methods.

There are two aspects of this technique, and others where nitrite plus nitrate are measured, that must be emphasized. First, the slurry activity represents a potential activity because conditions in the field, where the sample is obtained, may not be as conducive to nitrification as a shaken (i.e., aerated) slurry incubated at 25°C. Second, the activity measured is from the unenriched population. Since incubations are conducted over a short period, normally less than 25 h, little or no growth would occur.

The main reason for developing this technique is to assess the role of autotrophic nitrification in various natural environments. The accumulation of nitrite in the presence of chlorate, when compared with a control without chlorate, is direct evidence for the presence of autotrophic nitrite oxidation. The inhibition of nitrite (or nitrate) accumulation after the addition of nitrapyrin is direct evidence for autotrophic ammonium oxidation, since nitrapyrin is believed to be a specific inhibitor of autotrophic ammonium oxidation (9). This technique has been used to study a number of soils and sediments (manuscript in preparation). It was found that despite having low autotrophic MPN (most-probable-number)-countable populations, very high autotrophic activities existed.

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