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Nitrate Reduction to Nitrite, a Possible Source of Nitrite for Growth of Nitrite-Oxidizing Bacteria

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Growth yields and other parameters characterizing the kinetics of growth of nitrite-oxidizing bacteria are presented. These parameters were measured during laboratory enrichments of soil samples with added nitrite. They were then used to reanalyze data for nitrite oxidizer growth in a previously reported field study (M. G. Volz, L. W. Belser, M. S. Ardakani, and A. D. McLaren, J. Environ. Qual. 4:179-182, 1975), where nitrate, but not nitrite or ammonium, was added. In that report, analysis of the field data indicated that in unsaturated soils, the reduction of nitrate to nitrite may be a significant source of nitrite for the growth of nitrite oxidizers. A yield of 1.23×10^4 cells per μg of N was determined to be most appropriate for application to the field. It was determined that if nitrite came only from mineralized organic nitrogen via ammonium oxidation, 35 to 90% of the organic nitrogen would have had to have been mineralized to produce the growth observed. However, it is estimated that only about 2% of the organic nitrogen could have been mineralized during the growth period. Thus, it appears that another source of nitrite is required, the most likely being the reduction of nitrate to nitrite coupled to the oxidation of organic matter.

When ammonium is added to soil, autotrophic ammonium oxidizers and, subsequently, autotrophic nitrite oxidizers start to grow, with growth stopping when all of the ammonium is oxidized to nitrate. Alexander (2) has estimated that three times as many ammonium oxidizers, such as *Nitrosomonas europaea*, should be produced during this nitrification process as nitrite oxidizers, such as *Nitrobacter winogradskyi*. However, it is often observed that nitrite oxidizers are numerically more abundant in natural soils (15, 18).

In one case, it was observed that nitrite-oxidizing bacteria grew to high populations without the accompanying growth of ammonium-oxidizing bacteria when nitrate but no ammonium was added to a field (18). In that study it was concluded that there were two possible sources for the nitrite required for nitrite oxidizer growth. First, nitrite would be produced as a result of the oxidation of ammonium mineralized from soil organic nitrogen. In addition, nitrite could be produced as the result of nitrate reduction coupled to organic matter oxidation in anaerobic microenvironments, with the resulting nitrite diffusing into aerobic environments where it would be oxidized by the nitrite oxidizers. If ammonium oxidation were the major source, one would expect that growth of ammonium oxidizers would be required for growth of nitrite oxidizers to occur. This was not observed. After 3 weeks of the field study, nitrite oxidizer counts at depths of 30 and 60 cm were at least 100-fold higher than the ammonium oxidizer counts at the same depths. It was concluded on two grounds that oxidation of mineralized ammonium could not account for the growth of nitrite oxidizers: (i) nitrite oxidizers increased in number, whereas ammonium oxidizers did not; and (ii) more nitrite was required for the observed growth of nitrite oxidizers than could possibly have come from the oxidation of mineralized ammonium. The latter conclusion was based on growth yields, which were not given in the previous report (18) and are presented here with additional analysis of the field data.

MATERIALS AND METHODS

Hanford fine sandy loam was collected from the surface 5 cm of soil adjacent to a series of field plots described elsewhere (4, 18). The soil was air dry when collected. After collection the soil was sieved (1-mm mesh) and stored in an air-dry state until used. For reperfusion experiments the soil was stabilized by adding 0.4% Krilium by weight; the soil was then formed into 2-mm crumbs (5).

A buffered (1 mM potassium phosphate, pH 7.3) nutrient solution (containing 20 mg of $CaCl_2 \cdot 6H_2O$;

200 mg of $MgSO_4 \cdot 7H_2O$; 1 mg of 13% chelated iron [Geigy Chemical Corp., Newark, N.J.]; and trace elements of 0.1 mg of $NaMoO_4 \cdot 2H_2O$, 0.2 mg of $MnCl_2$, 0.002 mg of $CoCl_2 \cdot 6H_2O$, 0.1 mg of $ZnSO_4 \cdot 7H_2O$, and 0.02 mg $CuSO_4 \cdot 5H_2O$ per liter) was prepared and autoclaved at 15 lb/in^2 for 15 min. This was the basic microbial growth medium. In batch culture growth experiments, 25 ml of sterile growth medium containing 1 μmol of nitrite per ml was added to each of four shaker flasks. Two grams of $CaCO_3$ was added to two of these flasks; the pH of these flasks was 8.1. One gram of soil was inoculated into each flask, and the flasks were shaken for 16 days at 30°C. On day 10 of incubation, 200 μmol of nitrite was added to each. No nitrite was added to two control flasks (pH 7.3). Nitrite oxidizers were counted, and nitrite oxidized was measured from time to time.

Cyclic reperfusion apparatus were constructed from 20-mm-porosity B-filter funnels (90 mm in length) as columns; 250-ml flasks were reservoirs. The top of each column was capped with a stainless-steel cap. An inoculation needle was silver-soldered through each cap. A constant flow of nutrient was recycled from the resevoir to each column with the aid of a peristaltic pump (model 403, Laboratory Supply Co., Hickville, N.Y.). Twenty grams of Krilium-stabilized soil was contained in each apparatus. A 100-ml amount of solution was perfused at a temperature of 30 ± 1°C. Six apparatus were used; two were controls without nitrite. Nitrifier counts and nitrite oxidation were followed as a function of time. Five-gram subsamples of soil were taken from two of the growth flasks and from the two control flasks for making counts. Before taking a subsample, all of the soil was emptied from a reperfusion apparatus, excess moisture was removed with filter paper, and the soil was mixed thoroughly. After sampling, the remaining soil was added back to the same apparatus, and reperfusion was continued. As incubation proceeded, more nitrite was added to each apparatus (2,000 μmol of nitrite on day 10). After about 75 μmol of nitrite was oxidized per g of soil, the experiments were terminated, and populations of nitrifiers per gram were determined.

A nitrite oxidizer was isolated in pure culture by serially diluting (twofold dilutions into nitrite oxidizer growth medium) one of the positive tubes from the batch culture. Growth studies were done with this isolate and a pure culture of Engel's dark strain of *Nitrobacter* (obtained from N. Walker, Rothamsted Experimental Station, Herpenden, England). Growth flasks contained 100 ml of growth medium with nitrite at a 1.0 mM concentration. A 1% inoculum was used to inoculate duplicate flasks for each isolate. Incubations were carried out at 30°C. One mole of nitrite was added per flask when about 0.8 mmol of nitrite per ml had been oxidized.

Bacteria were counted by the most-probable-number (MPN) technique (1), using five tubes per dilution and 10-fold dilutions. Two counting media were used. One consist of the medium described by Alexander and Clark (3), which has a pH of 8.3. The other was the microbial nutrient medium, with a pH of 7.3 and a nitrite concentration of 0.1 mM.

Nitrite was measured by a modified Gries-Isovay procedure (8). Nitrate was measured periodically during the incubations by the chromotropic acid method (20). Most of the data obtained were based on the difference of nitrite concentrations. The accuracy of these measurements was confirmed by the periodic nitrate determinations.

The currently accepted practice for the classification of nitrite oxidizers (i.e., according to the 8th edition of *Bergey's Manual of Determinative Bacteriology* [19]) recognizes only one species of *Nitrobacter*, *N. winogradskyi*. However, it appears that there is a serological distinction between the previously recognized species *N. agilis* and *N. winogradskyi* (9) and that these strains may occupy different ecological niches (9). In this report the species *N. winogradskyi* will be considered to consist of two serologically distinct strains, which will be called *N. winogradskyi* and *N. agilis* to conform to previously used terminology.

Note that for certain calculations done in this report, data from a previous report (18) on organic nitrogen and carbon content of a soil profile are needed. These data are: for the 0- to 5-cm region, 219 μg of N per g and 2,020 μg of C per g; for the 30- to 35-cm region, 104 μg of N per g and 1,090 μg of C per g; and for the 60- to 65-cm depth, 71 μg of N per g and 870 μg of C per g.

RESULTS

Before considering measurement of growth and kinetic parameters, it is of interest to review some of the data obtained in the field study (18), during which it was observed that nitrite-oxidizing bacterial populations increased without an increase in ammonium-oxidizing populations. In Table 1, the data on nitrite oxidizer growth is given for maximum, median, and minimum counts at the three

TABLE 1. *Maximum, median, and minimum MPN counts for nitrite oxidizers determined during a field study, as a function of time and depth*[a]

Depth (cm)	Day	Cells per g		
		Maximum	Median	Minimum
0–5	0	1.5×10^5	5.3×10^3	8.2×10^2
	7	5.3×10^4	2.5×10^4	8.7×10^3
	14	2.8×10^6	3.5×10^5	5.4×10^4
	21	8.3×10^6	9.1×10^5	1.3×10^5
30–35	0	2.5×10^2	4.8×10^1	2.2×10^1
	7	2.7×10^3	2.5×10^2	2.2×10^1
	14	5.6×10^5	3.7×10^5	1.8×10^5
	21	9.0×10^5	5.4×10^5	2.5×10^5
60–65	0	<20	<20	<20
	7	22	<20	<20
	14	1.8×10^5	8.6×10^4	5.3×10^4
	21	2.8×10^7	8.0×10^5	1.5×10^5

[a] Data from field study by Volz et al. (18).

depths monitored in the soil profile. One can see by inspection that dramatic increases occurred in the populations at the 30- and 60-cm depths between days 7 and 14 of the study. Using the growth yields determined here, an estimate can be made of the amount of nitrite that must be oxidized to produce this observed nitrite oxidizer growth. The amount required can, in turn, be compared with the amount of nitrogen that can be released by mineralization of organic nitrogen to see if mineralization alone can account for the nitrogen required for the observed growth of these nitrifiers.

Growth yield measurements. Specific cell growth yields of nitrite-oxidizing bacteria indigenous to Hanford sandy loam were measured by two methods. One method consisted of measuring growth and oxidation characteristics in a batch culture of a soil suspension. The other method used a soil reperfusion technique.

Batch cultures were incubated at pH 8.1, with $CaCO_3$ added, and at pH 7.3. The latter pH value is the reaction of a Hanford sandy loam paste. Results of nitrite oxidation and nitrifier growth are plotted in Fig. 1 as functions of time. The amount of nitrite oxidized in the incubation flasks increased exponentially with respect to time; the same rate of oxidation was obtained at either pH. With the possible exception of day 15 of incubation, the MPN counts seemed to increase in proportion to the nitrite oxidized.

Growth studies were also done with the reperfusion technique at pH 7.3. Nitrite oxidation and measured bacterial growth are plotted in Fig. 2. Populations were estimated with counting solutions at pH values of 8.3 and 7.3. Both counts are plotted in Fig. 2. As in batch culture, nitrite disappeared at an exponential rate. Counts also appeared to have increased more or less exponentially with counting media of either pH 7.3 or pH 8.3. No growth was observed in control flasks, which had no added nitrite.

By dividing the cumulative amount of nitrite oxidized by a given day into the amount of cells produced by that day, specific cell yields can be computed. Yields calculated for batch culture and reperfusion studies are given in Tables 2 and 3, respectively.

There was considerable variation in the individual yields measured. There was more than a factor of 20 between the lowest yield measured and the highest. It appears that the batch culture yields were slightly higher than those measured in the reperfusion studies.

Organic N mineralization as a source of NO_2^- for NO_2^- oxidizer growth. From the data in Tables 2 and 3, a yield applicable to the field can be obtained. In the field study, the pH of the soil solution was 7.2, and the counts were made with a counting solution of pH 8.3.

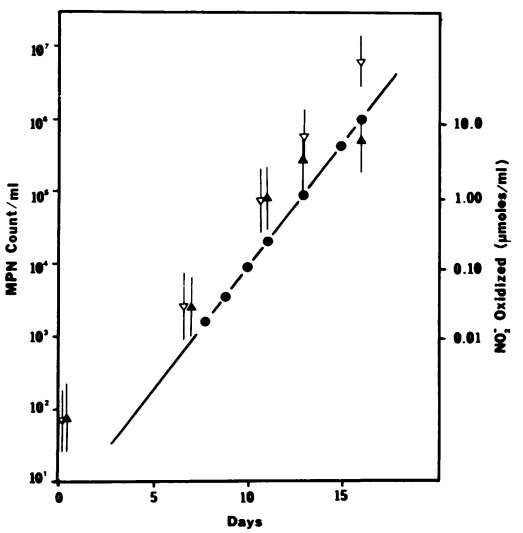

FIG. 1. *Growth of nitrite-oxidizing bacteria in batch culture. Symbols: ●, NO_2^- oxidized; ▲, counts with incubation flasks at pH 7.3; ▽, counts with incubation flasks at pH 8.1. Control counts, not given in figure, showed no growth. Note—all counts were done with a counting medium of pH 8.3.*

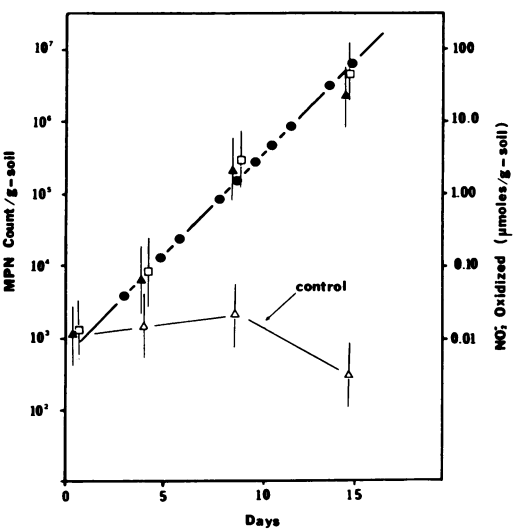

FIG. 2. *Growth of nitrite oxidizers during reperfusion experiments. Symbols: ●, NO_2^- oxidized; ▲, counts with a pH 8.3 counting medium; □, counts with a pH 7.3 counting medium; △, control counts with counting medium of pH 8.3.*

TABLE 2. *Measurement of growth yields and maximum oxidizing rate per bacterium under conditions of exponential growth, using batch culture techniques*

pH		Incubation time (days)	NO_3^- produced (μmol/ml)	NO_2^- oxidizer count (cells/ml)	Yield (cells/μg of N)	k (pmol/h·cell)
Soil	Count					
8.1	8.3	11	0.20	$7.0 \times 10^4 \pm 4 \times 10^4$	2.8×10^4	0.090
		13	0.95	$5.6 \times 10^5 \pm 2 \times 10^5$	4.2×10^4	0.054
		16	10.9	$5.6 \times 10^6 \pm 3 \times 10^6$	3.6×10^4	0.062
7.3	8.3	11	0.19	$8.9 \times 10^4 \pm 4 \times 10^4$	3.1×10^4	0.068
		13	0.95	$2.9 \times 10^5 \pm 1 \times 10^5$	2.2×10^4	0.10
		16	10.7	$4.1 \times 10^5 \pm 2 \times 10^5$	2.6×10^3	0.84

TABLE 3. *Measurement of growth yields and maximum oxidizing rate per bacterium under conditions of exponential growth, using reperfusion techniques*

pH		Incubation time (days)	NO_2^- produced (μmol/g)	NO_2^- oxidizer count (cells/g)	Yield (cells/μg of N)	k (pmol/h·cell)
Soil	Count					
7.3	8.3	4	0.075	$6.0 \times 10^3 \pm 3 \times 10^3$	5.6×10^3	0.33
		9	1.6	$2.4 \times 10^5 \pm 1 \times 10^5$	1.1×10^4	0.17
		16	75	$1.9 \times 10^6 \pm 3 \times 10^6$	1.8×10^3	1.10
7.3	7.3	4	0.072	$8.1 \times 10^3 \pm 3 \times 10^3$	7.6×10^3	0.24
		9	1.6	$4.0 \times 10^5 \pm 2 \times 10^5$	1.7×10^4	0.10
		16	76	$5.4 \times 10^6 \pm 2 \times 10^6$	4.7×10^3	0.39

When only data obtained under these conditions are used in the yield average, a value of 1.23×10^4 cells per μg of N (standard error, 0.48×10^4) is obtained.

In Table 4 the amounts of nitrogen required to produce median and minimum changes in the field populations of nitrite oxidizers at the three depths (from Table 1) are given as percentages of organic nitrogen at those depths. According to these calculations, between 35 and 91% of the organic N would have to be mineralized to account for the median counts, and between 5 and 20% would be required to account for the minimum counts.

Estimates for the rate of organic N mineralization can be calculated from data in the literature. Broadbent and Nakashima (6) measured organic N mineralization rates for barley roots added to Colombia silt loam. From their data, it appears that the nitrogen was mineralized from the added material at a rate of 0.08 to 0.10% per day, with the rate for organic N indigenous to soil being a factor of 3 lower. With these values, a range of 0.6 to 2.1% of the organic N would be mineralized during the 3-week growth period in the field. Keeney and Bremner (10) measured the amount of mineralizable N during 3-week incubation studies in 10 midwestern soils. An average of 2.1% (standard error, 1.3%) of the organic N was mineralizable. The highest value calculated was 4.8%. None of these rates appear to be sufficient to account for the observed growth.

TABLE 4. *Calculated percentages of soil organic N that would have to be mineralized or soil organic C that would have to be oxidized, coupled to nitrate reduction to nitrite, to produce the observed growth of nitrite oxidizers in a field study*

Depth (cm)	Growth requirement (%)[a]			
	Organic N mineralization		Organic C oxidation	
	Median	Minimum	Median	Minimum
0–5	33.8	4.83	1.57	0.22
30–35	42.2	19.5	1.73	0.80
60–65	91.5	17.2	3.20	0.60

[a] Calculations based on counts in Table 1.

NO_3^- reduction as a source of NO_2^- for NO_2^- oxidizer growth. One can also calculate the amount of organic carbon that might have been metabolized if the population were growing on nitrite produced by nitrate reduction. The Mebius method (13), used to measure oxidizable carbon in the field (18), measures carbon in micrograms per gram, assuming an average oxidation state for carbon of zero (carbon atoms in carbohydrates have an average oxidation state of zero). On this basis the following equation for nitrate reduction can be written: $CH_2O + 2NO_3^- \rightarrow CO_2 + 2NO_2^- + H_2O$. From this equation ones sees that the oxidation of one atom of carbon can reduce two atoms of nitrogen; thus, 12 μg of C would reduce 28 μg of N as NO_3^-. Using this relation, the percentage of oxidizable carbon that would

be required to produce the observed nitrite oxidizer growth can be calculated. These values are also tabulated in Table 4. The requirement for growth of the average population with nitrite from this source is less than 3.2% of the oxidizable carbon, with the minimum population growth requiring less than 0.8% of the carbon. These values seem reasonable for the total amount of organic carbon that could be oxidized during the 3-week growth period. However, more oxidation than is indicated by these estimates would probably be required, since it is not likely that all of the organic carbon oxidation would be coupled to nitrate reduction in an unsaturated soil.

Possible counting inefficiencies. On day 15 of the batch culture study incubated with a pH of 7.3, the counts (duplicate) were lower than expected (i.e., a factor of 10 different from the counts obtained for the pH 8.1 incubation). Note that at both incubation pH values, the oxidation rates were essentially the same on any day and increased exponentially at the same rate. It seems reasonable to assume that biomass and cell counts would increase exponentially also. The MPN method, as used here, has a 95% confidence limit of a factor of 3.3 per count (1), if all of the cells were counted. Since the variation was much greater than this and the variation between duplicated counts was less than a factor of 2, it seemed possible that the pH values of the counting media may have affected the counting efficiency.

With a pure culture isolated from one of the batch culture growth flasks, a study was done to see the effect of counting medium pH on counting efficiency. Figure 3 shows the oxidation kinetics and bacterial counts of this isolate as functions of time when grown at pH 7.3. Counting media of pH values of 7.3 and 8.3

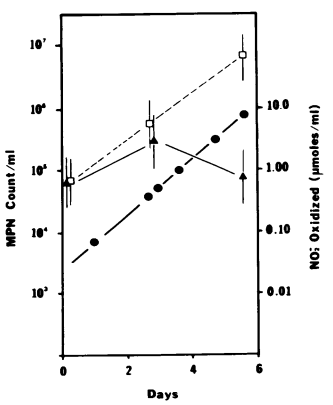

FIG. 3. *Growth of N. agilis strain isolated from Hanford sandy loam. Symbols: ●, NO_2^- oxidized; ▲, counts with a pH 8.3 counting medium; □, counts with a pH 7.3 counting medium.*

were used. It appears that the pH 8.3 medium did not effectively count this isolate. In similar growth studies with Engel's dark strain of *Nitrobacter*, no difference in counting efficiency between the two media was observed. Thus, the problem appears to be a function of the strain or species present. The Hanford sandy loam isolate has subsequently been identified as *N. agilis* by E. L. Schmidt, University of Minnesota, by fluorescent antibody techniques (9). From Fig. 3 (using the data obtained with pH 7.3 counting medium) a yield of 6.9×10^4 cells per μg of N as NO_2^- and a generation time of 16.9 h are obtained. With similar growth conditions Engel's dark strain of *Nitrobacter* grew with a generation time of 8.25 h and a yield of 3.3×10^5 cells per μg of N.

Kinetic considerations. Of all the data obtained during the growth studies, the measured product (NO_3^-) formation appears to have had the greatest precision. This is implied by the straight line obtained from plotting log product (P) versus time (t) in Fig. 1 and 2. Such plots imply that exponential growth is taking place. An apparent growth rate constant, γ, can be determined for both batch and reperfusion studies, using only product formation data, with equation 1: $dP/dt = \gamma P$. This is simply a form of the Monod growth equation (14), where the biomass (or bacterial cell count) in his equation is replaced by P. P is the amount of substrate that must be oxidized to produce a given population, when the growth yield (Y) is constant (i.e., YP = bacterial cell count). The integrated form of this equation has been shown to hold for a nitrifier by Buswell et al. (7). A value of 0.032/h was obtained for γ in batch culture (generation time, 21.7 h) and a value of 0.026/h was obtained in the reperfusion studies (generation time, 26.7 h).

The rate at which nitrate is being produced at any time during incubation is given by equation 1, and this rate can be used to calculate the nitrate oxidation rate per cell during exponential growth in the following way. Since the rate of nitrite oxidation is simply the negative of nitrate production, the rate of nitrite oxidation per cell, k, is obtained by dividing the cell count (m) at a given time into the product of the specific growth rate and the nitrate concentration at that time: $k = \gamma P/m$ (equation 2). As was seen with the growth yields, there is considerable variation in the calculated activities per cell (Tables 2 and 3). When k is large, the corresponding value for Y is low. Such an inverse relationship is expected, since k is proportional to $1/Y$. k also varies with γ ($k = \gamma P/m = \gamma/Y$), but since the γ's measured in the two soil studies show only a slight variation, it appears that this factor makes little contribu-

tion to the variation in k.

Kinetic analyses of the field data. Although growth yield-mineralization rate calculations give important information on the limits of population increase that could occur within a given period, they give no information on whether the population can fulfill that potential.

There are two growth phases of nitrite oxidizers in the field that should be noted. During the initial phase of growth, when the populations are small, nitrite is produced at a rate faster than it can be oxidized, and nitrite will accumulate, allowing the population to grow exponentially. At some point during the growth period, the rate of nitrite oxidation required to maintain exponential growth will become larger than the rate at which nitrite is being produced. At this point the nitrite concentration in the soil will start to decline. When the nitrite concentration is reduced sufficiently, the growth rate will decrease due to substrate limitation.

In the field study, nitrite reached its maximum in soil solution at about 10 days. It may be assumed that at this point the rate of nitrite production approximately equalled the amount required for exponential growth. By the end of the second week the majority of the nitrite required for growth must come directly from nitrite production and not from depletion of the nitrite pool. This is seen by noting that at no depth was the concentration of nitrite above 0.012 μg of N per g of soil (0.10 μg/ml in soil solution). This is insufficient to produce even 200 cells per g if the pool were entirely depleted. Cell production was a factor of at least 1,000 higher. Thus, the nitrite oxidation rate (and thus growth) must be limited by the rate at which nitrite is produced.

By manipulating the Monod growth equations (14) the following general growth equation can be obtained: $-dS/dt = dP/dt = m\gamma/Y$. In this case it is assumed that only an insignificant amount of substrate, S, is incorporated in biomass. It should be noted that neither γ nor Y are constants and that, in fact, they are not even independent. For example, as γ approaches zero, Y will also approach zero. However, dP/dt does not necessarily have to approach zero, since oxidation may be required to maintain biomass (17) or uncoupled from useful processes and wasted (11).

If it is assumed that by the end of the second week dP/dt has reached a maximum value equal to the organic N mineralization rate (0.1%/day), as argued above, and that the cells are produced with a yield of 1.23×10^4 cells per μg of N, an estimate can be made of how large the population will be when their generation time has slowed to 168 h (1 week). These estimates are 2.7×10^4 cells per g for the 0- to 5-cm depth, 1.3×10^4 cells per g for the 30- to 35-cm depth, and 8.8×10^3 cells per g at the 60- to 65-cm depth. After the populations reach these sizes, it would take them 1 week or longer to double. These populations are about 70% of the population sizes that would be estimated using 1.4% mineralization of organic N during a 2-week period.

Since the yield used in these calculations (1.23×10^4) is applicable only to cultures growing with a 20- to 30-h generation time, it is of interest to see how a correction for maintenance requirement (17) might affect these estimates. Using data obtained by C. Chiang (Ph.D. thesis, Université Catholique de Louvain, Louvain, Belgium, 1969) for maintenance requirements of *Nitrobacter*, it can be estimated that only 35% of the bacteria produced with a 20- to 30-h generation time will be produced with a generation time of 168 h, per μg of N as NO_2^-. Thus, the populations calculated above may be overestimated by a factor of 3. It seems reasonable to conclude that the nitrite oxidizers cannot fulfill their growth potential as estimated by multiplying the net mineralization (3 weeks) times cell yield, if mineralization is the only source of nitrate, since limited substrate availability limits both growth rate and yield efficiency.

DISCUSSION

The analysis of the field data presented here supports the conclusions reached previously (18) that growth yield data support the hypothesis that mineralization of organic N alone can not provide enough nitrite (via ammonium oxidation) to account for the high populations of nitrite oxidizers. The MPN count data, on which the main conclusion is based, suffer from certain inadequacies, which are enumerated in the following discussion. However, none of these problems, taken singly or in sum, are sufficient to invalidate the conclusion stated above.

All arguments presented here depend on the accuracy with which the yields could be measured. It is apparent that there is a large variation in the values obtained (Tables 2 and 3). In addition, the two pure culture studies gave values that were larger than any of the values measured in soil, and the yields measured for these two cultures appear to be significantly different.

If it is assumed that the MPN counting procedure efficiently counted all of the *Nitrobacter* cells present and that neither nitrite

nor oxygen was limiting, the difference in the two pure culture yields may be attributed to two causes. First, it appears that the Hanford isolate may have a larger biomass. This can be seen if the maximum activities per cell are compared. The activity, k, of the Hanford isolate (0.042 pmol/h per cell) is more than twice the activity of the Engel isolate (0.018 pmol/h per cell). If it is assumed that biomass is in some way proportional to the maximum enzyme activity per cell, one can assume that the decrease in cells produced per microgram of N oxidized is partly due to the production of larger-biomass cells. A second feature that may account for the lower yield of the Hanford isolate is its relatively long generation time (16.9 h), which is twice as long as the generation time for Engel's strain (8.25 h). As mentioned previously, the amount of substrate oxidized per generation required for maintenance increases with increasing generation times, which reduces the yield. If the metabolism required to maintain a unit of biomass for a unit time is the same for both nitrifiers, roughly twice as much metabolism will be required to maintain a unit of biomass for the Hanford isolate per generation as for the Engel isolate (since the generation time is twice as long). In addition, if the Hanford isolate has 2.25 times the biomass of the Engel isolate per cell, as assumed above, the maintenance requirement per generation would be about 4.5 times higher for the Hanford isolate than that for the Engel isolate. It seems possible that an increase in biomass and an increase in maintenance requirement per generation can account for the decrease in yield of the Hanford isolate when compared with the yield of Engel's strain.

The difference between the yield determined in soil studies and that determined for the Hanford isolate is probably due mainly to the inability to desorb all the nitrifiers from the soil particles. With the MPN counting technique, if not all the bacteria are desorbed and several bacteria adhere to the same particle, each particle would count as only a single bacterium. This will lower the apparent count. The highest yields for the soil incubations presented here were determined in a batch culture of a soil suspension. It is likely that more cells were growing in a free, desorbed state under these conditions than was the case in the reperfusion experiments, increasing the counting efficiency. These soil suspension values approach the yield determined for the Hanford soil isolate in liquid culture, where adsorption isn't a problem. In addition, in the soil studies the generation times were longer than those for the Hanford isolate, which would tend to decrease the yield, as mentioned above. Yield values for nitrite oxidizers in soil can be calculated as 2.2×10^3 cells per μg of N, 1.5×10^3 to 2.0×10^3 cells per μg of N, and 4.0×10^3 to 9.0×10^3 cells per μg of N from the work of Morrill and Dawson (15), Nishio and Furusaka (16), and R. Rennie and E. L. Schmidt (Ecol. Bull. [Stockholm], in press), respectively. The first two studies were done with reperfusion and MPN counting techniques, whereas the latter was done with static incubations of six soils, using fluorescent antibody counting techniques. In comparison with these values, the value of 1.23×10^4 cells per μg of N obtained in this study doesn't appear to be unrealistically low, even though it is significantly lower than those obtained in the pure culture studies. It is not clear why the yields measured in the work presented here are higher than those calculated above. Perhaps the difference is the result of an inadequate incubation period. Nishio and Furusaka (16) and Morrill and Dawson (15) used an incubation period of 3 weeks (or slightly longer), whereas an incubation period of 5 weeks or longer was used in the work presented here. Data obtained by Matulewich et al. (12) indicate that counts can be increased 6- to 10-fold by extending the incubation time this much. In fact, according to Matulewich et al. (12), 5 weeks of incubation may not be adequate.

In this work it is also assumed that the yield determined in the laboratory studies is directly applicable to the field. Thus, one must assume that the laboratory incubations enrich the same nitrite oxidizers that are proliferating in the field. Recent evidence (Rennie and Schmidt, Ecol. Bull. [Stockholm], in press) indicates that both *N. winogradskyi*- and *N. agilis*-type strains do coexist in field soils. Other evidence indicates that *N. winogradskyi*-type strains may be the faster growing of the two (9). An *N. agilis*-type strain is likely to be the predominant strain in the soil incubations, since such a strain was isolated from one of the flasks at the end of the study. These soil incubations appear to have generation times nearly twice as long as the apparent generation times of field populations at subsurface depths during the second week of the field study. In the field, generation times, based on median counts, were 14 to 16 h, whereas in the incubation studies, they were 21.7 to 26.7 h. It is not clear what the significance of this is, but if a different strain or species population occurred in the surface soil from that in the subsurface regions, then yields determined in soil incubations that used surface soil only would not necessarily be applicable to subsurface growth.

It is also apparent from the pure culture growth studies presented here that different strains of *Nitrobacter* can have significantly different yields. Although this possibility affects the calculations presented here for estimated population sizes, it doesn't change the original conclusion that mineralization of organic N cannot account for the growth of the nitrite oxidizers in this field study (18). The yield would have to be larger by a factor of at least 20 to 25 to negate these conclusions (i.e., to reduce the required mineralization at the 60- to 65-cm depth [Table 4] from 91.5 to 3.5% would require a 25-fold increase in yield). It seems unlikely that the difference in yield could be larger than a factor of 5, which is the difference between the yield determined for Engle's dark strain of *Nitrobacter* and the yield for the Hanford isolate of *Nitrobacter*. The additional difference (decrease) in yield seen in pure culture versus soil studies is due mainly to my inability to desorb all the nitrifiers from soil particles, as discussed earlier.

It should be noted that although it is likely that populations were underestimated in the laboratory study, it is equally likely that the experimentally determined populations were underestimated by the same ratio. Thus, it would appear that the conclusion that field populations were too large to be accounted for by oxidation of mineralized organic N is valid. Hence, some other source, presumably the reduction of nitrate coupled to oxidation of organic matter, must be involved.

From this analysis, it appears that the ecology of nitrite-oxidizing bacteria is complex, and the existence of large populations of nitrite-oxidizing bacteria in a soil sample is not necessarily indicative of active nitrification of ammonium taking place concurrently. It is clear also that a better understanding of soil microenvironments is necessary before a good understanding of nitrogen cycling in unsaturated soils can be achieved. None of the transformations hypothesized here (nitrate reduction to nitrite and its subsequent oxidation) have been measured explicitly. The sole evidence remains the data published previously (18), that nitrite oxidizers grew without accompanying growth of ammonium oxidizers and that the amount of nitrite that could be produced as a result of organic N mineralization cannot account for this growth.

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