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Inhibition of Ammonium Oxidation by Nitrapyrin in Soil and Liquid Culture

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Inhibition of growth of axenic cultures of *Nitrosomonas europaea* by nitrapyrin was investigated in liquid culture and in soil. In liquid culture, exponentially growing cells were more sensitive than stationary-phase cells, possibly due to a requirement for uptake of nitrapyrin, metabolism of nitrapyrin, or both before inhibition. Differences in sensitivity were observed between the parent strain and two strains, *sp1* and *sp2*, that were selected through repeated subculturing. These differences were reflected in the length of the lag period induced by nitrapyrin and in the specific growth rate and were due to different bactericidal and bacteriostatic effects. Soil provided significant protection from inhibition, with concentrations of nitrapyrin approximately one order of magnitude greater than those required for equivalent inhibition in liquid culture. The data show that strain differences alone do not explain differences in sensitivity between nitrification in soil and in liquid culture and suggest that the inhibitor may be more effective against actively nitrifying soils.

Nitrification is the two-stage microbial oxidation of ammonium to nitrate via nitrite. The first stage is carried out by autotrophic ammonia-oxidizing bacteria, such as *Nitrosomonas*, *Nitrosospira*, and *Nitrosolobus* species; and the second stage is carried out by nitrite oxidizers, usually *Nitrobacter* species. Inhibition of nitrification can improve the nitrogen status of soil by reducing the loss of ammonia-N to nitrate, which is then leached or lost to the atmosphere by denitrification. The rate-limiting step in nitrification is generally the oxidation of ammonium, and nitrate rarely accumulates in soil. Commercial inhibitors of nitrification are therefore specific inhibitors of ammonium oxidation. One such inhibitor is nitrapyrin [2-chloro-6-(trichloromethyl) pyridine] (6), which is thought to exert its inhibitory effect by chelating the copper components of enzymes involved in ammonia oxidation (4, 8), although little research has been carried out into the biochemical mechanism of inhibition.

The sensitivity of autotrophic ammonia oxidizers to nitrapyrin has been found to differ in soil and liquid culture (15). This difference has been attributed in part to a number of factors including adsorption and inactivation of the inhibitor by organic matter (3, 6, 11), pH and temperature effects (2, 3, 7), and differences in ammonia oxidizer strains and their sensitivities to the inhibitor (1).

Similar differences in sensitivities have also been reported with other inhibitors of nitrification, e.g., herbicides (14) and potassium ethyl xanthate (18). With the exception of the latter, all studies of inhibition in soil have involved the natural soil microflora, preventing meaningful conclusions about the significance of strain differences. The aim of this study was to compare inhibition in pure cultures of ammonia oxidizers with that in sterile soil inoculated with the same cultures, to eliminate interactions with other microorganisms and strain differences as possible explanations for differences in sensitivity.

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MATERIALS AND METHODS

Axenic cultures of a soil isolate of *Nitrosomonas europaea* were used in all experiments. Details of its source and routine maintenance are given elsewhere (13). Repeated subculturing led to selection of two strains of *N. europaea*, termed *sp1* and *sp2*. Both were obtained from the parent strain, and the same conditions and media were used for the maintenance of all three strains. Growth in liquid culture was studied in 250-ml Erlenmeyer flasks containing 150 ml of modified medium (13) described by Skinner and Walker (17) inoculated with 2 ml of a stationary-phase culture of *Nitrosomonas europaea*, incubated at 30°C, and shaken at 150 rpm. The initial ammonium concentration was 50 μg of NH_4^+-N ml^{-1} as ammonium sulfate, and triplicate flasks were used for each treatment. Samples were removed at frequent intervals, stored at −15°C, and analyzed for ammonium-N and nitrite-N with an Autoanalyser II (Technicon Instruments Corp., Tarrytown, N.Y.). Sampling continued until acid produced by ammonium oxidation decreased the pH significantly from the initial value of 8, causing a decrease in specific growth rate. This occurred when the nitrite concentration reached approximately 8 μg ml^{-1}. Nitrapyrin was added at final concentrations in the range of 0 to 1 μg ml^{-1} either immediately prior to inoculation or to exponentially growing cultures, with the latter being assessed as those in which the nitrite concentration reached approximately 2 μg ml^{-1}. Nitrapyrin was prepared by emulsification in 0.5% ethanol which was then diluted in distilled water. The solution was filter sterilized before it was added to culture media.

Viable counts. Viable cell concentration was estimated by the most probable number (MPN) method. Samples (1 ml) were taken from exponentially growing cultures before and after the addition of nitrapyrin, and 1/10 serial dilution series were constructed in ammonium-free medium described by Skinner and Walker (17). Five 1-ml fractions were transferred from each dilution to five replicate tubes containing 9 ml of medium described by Skinner and Walker (17). Tubes were then incubated at 30 ± 2°C in the dark for 28 days, after which growth was assessed by spot tests for nitrite with

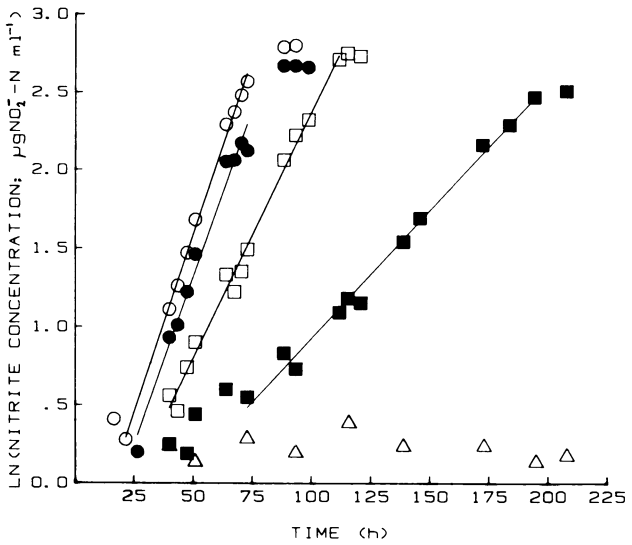

FIG. 1. Production of nitrite during growth of *N. europaea* in liquid batch culture following the addition of 0 (○), 0.2 (●), 0.5 (□), 0.7 (■), and 1.0 (△) μg of nitrapyrin ml^{-1} to stationary-phase cells. Lines of best fit during exponential nitrite production were used to calculate specific growth rates. Data are presented from one flask of a set of three replicate flasks.

Griess Ilosvay reagents I and II. The MPN of *Nitrosomonas* cells was calculated from statistical tables (12).

Soil incubation studies. Ammonium oxidation in soil was studied in 250-ml Erlenmeyer flasks containing 80 g of air-dried soil. Soil was autoclaved at 121°C for 30 min, and 0.44 g of sterile calcium hydroxide was added to adjust the soil pH to 8. Each flask was also provided with 20 ml of sterile solution of ammonium sulfate (50 μg of NH_4^+-N ml^{-1}) and an inoculum of 2 ml from a stationary-phase culture of *N. europaea spl*. Nitrapyrin was prepared as described above, and 1 ml was added aseptically to give a final concentration in the range of 0 to 7.5 μg ml^{-1}. Soil was thoroughly mixed and incubated at 30°C in the dark. Triplicate flasks were prepared for each treatment.

Soil samples (approximately 2.5 g) were removed aseptically at regular intervals, and ammonium and nitrite were extracted by shaking for 25 min with 12.5 ml of 2 M KCl. The suspension was filtered, and the filtrate was stored at −15°C before analysis for ammonium and nitrite as described above. Flasks were weighed regularly, and evaporation losses were corrected by the addition of sterile distilled water.

Hydrolysis of nitrapyrin. Hydrolysis of nitrapyrin was determined by the method of Bremner et al. (2). Nitrapyrin was added to 150 ml of medium (pH 8) described by Skinner and Walker in 250-ml Erlenmeyer flasks to give final concentrations of 30, 50, and 70 μg ml^{-1}. Flasks were incubated at 30°C and 150 rpm. At regular intervals, 10-ml samples were removed and shaken with 5 ml of 0.5 M sodium bicarbonate and 10 ml of hexane (Analar grade, redistilled) in a 125-ml separating funnel. Nitrapyrin enters the hexane because of its hydrophobic nature, and adsorption of the hexane phase was measured at a wavelength of 270 nm. Nitrapyrin concentrations were calculated from a standard curve in the range of 0 to 100 μg ml^{-1}.

Measurement and comparison of specific growth rates. Triplicate flasks were set up for each treatment, and the specific growth rate was determined for each individual flask. Data for each treatment were not pooled for calculation of the specific growth rate because of variability in the length of the lag phase. Growth rates for each triplicate flask were then used to calculate the mean specific growth rate for a particular treatment. The standard error (SE) associated with each individual growth rate was much lower than the SE of the mean, and comparisons of specific growth rates from the different treatments were made on the means of triplicate flasks by the standard Student *t* test with pooled variance.

RESULTS

Liquid culture. The effect of the addition of a range of nitrapyrin concentrations, prior to inoculation, on growth in liquid culture is illustrated in Fig. 1. In the absence of nitrapyrin there was a lag phase of 21 h before growth commenced, followed by exponential growth at a specific rate of 0.045 h^{-1}. The addition of nitrapyrin increased the length of the lag phase, and the specific growth rate decreased with increasing inhibitor concentration. Specific growth rates and lag periods are summarized in Table 1, along with results of the Student *t* test statistics, which compared specific growth rates with those of the control. A concentration of 0.2 μg of nitrapyrin ml^{-1} did not significantly reduce the specific growth rate, although it did slightly increase the duration of the lag phase. The specific growth rate was significantly decreased at concentrations greater than 0.5 μg ml^{-1}, and the lag period increased further at these higher concentrations. No growth occurred within 4 weeks following the addition of 1 μg of nitrapyrin ml^{-1}.

Following the addition of 0.5 μg of nitrapyrin ml^{-1} to exponentially growing cultures, production of nitrite was immediately inhibited and an apparent lag phase was induced, which lasted for 70 h before growth resumed at a reduced rate (Fig. 2). The specific growth rate following the addition of nitrapyrin was 0.0076 h^{-1}, which is significantly lower than 0.045 h^{-1}, the control rate (t = 12.7; P = 1.00), and 0.033 h^{-1}, the rate following the addition of inhibitor to stationary-phase cultures (t = 11.24; P = 1.00).

Laskowski and Bidlack (9) and Rodgers and Ashworth (15) reported that nitrapyrin killed a percentage of the ammonia-oxidizing population, while it had a bacteriostatic effect on the surviving cells. Therefore, the lag induced following the addition of 0.5 μg of nitrapyrin ml^{-1} to growing cultures could be due to a reduction in the size of the viable ammonia-oxidizing population. This is supported by MPN counts prepared before and after addition, which showed a reduction in viable cell concentration from $2.4 \times 10^5 \pm 1.1 \times 10^5$ (SE) cells ml^{-1} to $<1 \times 10^5$ cells ml^{-1} 22 h after addition. Control flasks showed an increase from $3.5 \times 10^5 \pm 0.52 \times 10^5$ (SE) cells ml^{-1} to $4.44 \times 10^5 \pm 1.11 \times 10^5$ (SE) cells ml^{-1} during the same period.

If nitrapyrin kills a proportion of cells and the remaining population continues to oxidize ammonia at an unaltered

TABLE 1. Effect of nitrapyrin on the specific growth rate and the length of lag period of *N. europaea*

Nitrapyrin concn (μg ml^{-1})	Specific growth rate (h^{-1})	Length of lag period (h)	Degrees of freedom	t^a	P
0.0	0.045	22			
0.2	0.043	25	14	1.05	0.69
0.5	0.033	33	17	8.13	1.00
0.7	0.016	64	16	22.86	1.00

[a] Results of the Student *t* test.

rate, the net change in nitrite concentration per unit time will be less than that before nitrapyrin addition. A semilogarithmic plot of nitrite concentration versus time would then show an apparent reduction in specific growth rate, similar to that observed in Fig. 2. The proportional change in nitrite concentration, from which the specific growth rate is calculated, will be unaltered only if this reduction in viable cell concentration is taken into account. The true specific growth rate therefore can be calculated by subtracting the nitrite concentration at the time of the addition of nitrapyrin from all subsequent concentrations. The specific growth rate is then represented by the equation $\mu = [\ln (NO_2)_t - \ln (NO_2)_0]/t$, where μ is the specific growth rate, and (NO_2) and $(NO_2)_t$ are nitrite concentrations at the time of nitrapyrin addition and at a time t after addition, respectively. Application of this equation to the data shown in Fig. 2 gives a corrected specific growth rate of 0.0070 h^{-1}, which is not significantly different from the apparent rate of 0.0076 h^{-1} (t = 0.49; P = 0.36). The observed reduction in viable cell concentration was therefore insufficient to reduce the specific growth rate, and the lag phase was due to a bacteriostatic effect.

Routine maintenance of *N. europaea* was achieved by subculturing in liquid medium at 14-day intervals. This presents the possibility for the selection of strains with a particular advantage under the conditions that were used for maintenance. For example, soil isolates might be expected to have high substrate affinities, rather than high maximum specific growth rates. Continued subculturing in medium containing high initial substrate concentrations will provide no advantage for strains with low K_s values but will select for those with high maximum specific growth rates. All culturing was carried out in inorganic medium containing 50 μg of NH_4^+-N ml^{-1}, in the absence of nitrapyrin, and all strains were maintained under the same conditions. During the course of this study two such strains were obtained, with growth characteristics different than those of the original strain. Growth of the first of these, strain *sp1*, and the effect of the addition of 0.5 μg of nitrapyrin ml^{-1} to exponentially growing and stationary-phase cultures is illustrated in Fig. 3.

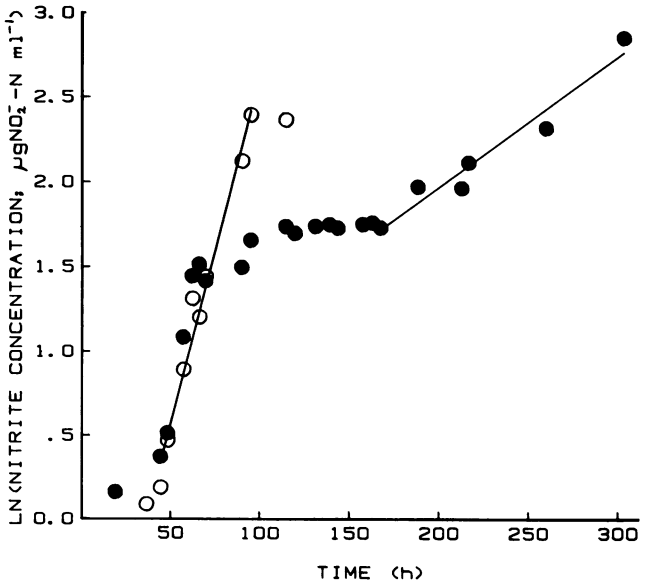

FIG. 2. Production of nitrite during growth of *N. europaea* in liquid batch culture with (●) and without (○) the addition of 0.5 μg of nitrapyrin ml^{-1} during exponential growth (60 h after inoculation). Lines of best fit were used to calculate specific growth rates. Data are presented from one flask of a set of three replicate flasks.

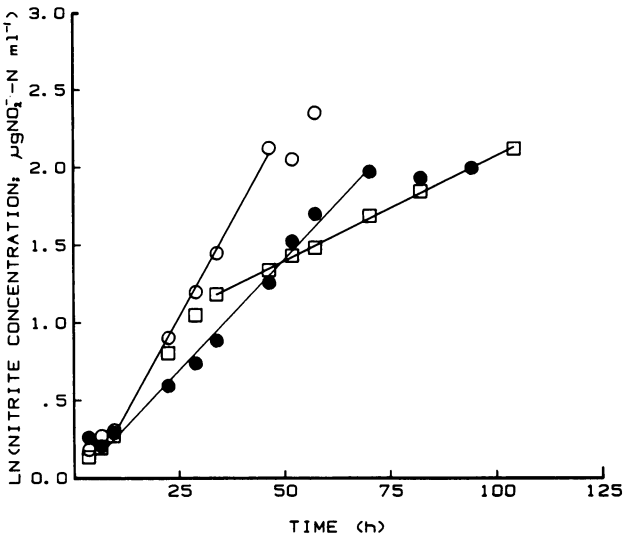

FIG. 3. Production of nitrite during growth of *N. europaea sp1* in liquid batch culture in the absence of nitrapyrin (○) and following the addition of 0.5 μg of nitrapyrin ml^{-1} at 0 h (●) and during exponential growth (27 h after inoculation) (□). Lines of best fit were used to calculate specific growth rates. Data are presented from one flask of a set of three replicate flasks.

In the absence of nitrapyrin, growth commenced after a lag phase of approximately 8 h and proceeded at a specific rate of 0.047 h^{-1}, which is not significantly different than that of the original strain (t = 0.70; P = 0.50). Following the addition of nitrapyrin to stationary-phase cells, the lag phase was not increased but the specific growth rate was reduced to 0.029 h^{-1}, a similar reduction to that found in the original strain. The addition of nitrapyrin to exponentially growing cells produced a greater reduction in specific growth rate to 0.0133 h^{-1}, but this reduction was less than that observed in the original strain and a lag phase was not induced. In addition, viable cell concentration did not decrease following the addition of nitrapyrin but increased at a reduced rate (Table 2). It is therefore unlikely that the reduction in the specific growth rate following the addition of nitrapyrin to exponentially growing cells of strain *sp1* was due to a reduction in the size of the viable population. This is confirmed by the close similarity between the apparent specific growth rate (0.0133 h^{-1}) and the corrected rate (0.0134 h^{-1}) (t = 0.03; P = 0.02).

A second, fast-growing strain, *sp2*, was also isolated. In

TABLE 2. Effect of the addition of 0.5 μg of nitrapyrin ml^{-1} on MPN of *N. europaea sp1* when added to exponentially growing cultures 27 h after inoculation[a]

Time following inhibitor addition (h)	MPN of cells ml^{-1}	
	Without nitrapyrin	With nitrapyrin
0	$2.3 \times 10^5 \pm 0.05 \times 10^5$	$2.3 \times 10^5 \pm 0$
2	$2.9 \times 10^5 \pm 0.55 \times 10^5$	$3.3 \times 10^5 \pm 0$
30.5	$28.5 \times 10^5 \pm 6.5 \times 10^5$	$6.0 \times 10^5 \pm 1.05 \times 10^5$

[a] Data are presented as mean ± SE.

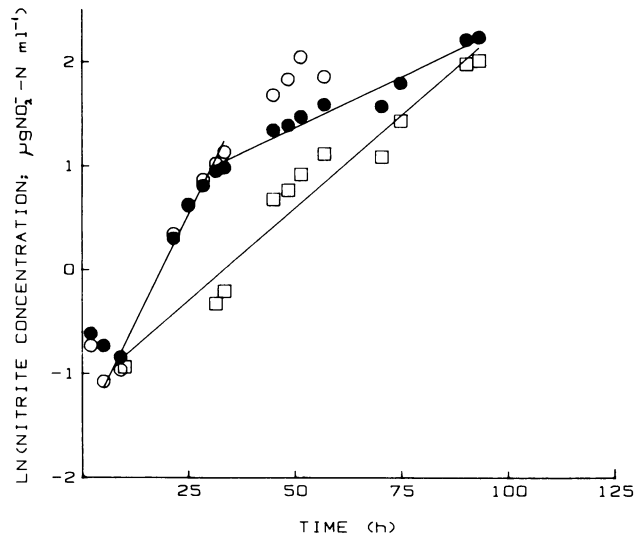

FIG. 4. Production of nitrite during growth of *N. europaea sp2* in liquid batch culture in the absence of nitrapyrin (○) and following the addition of 0.5 μg of nitrapyrin ml^{-1} at 0 h (□) and during exponential growth (27 h after inoculation) (●). Lines of best fit were used to calculate specific growth rates. Data are presented from one flask of a set of three replicate flasks.

the absence of nitrapyrin this strain had a specific growth rate of 0.083 h^{-1}, which is significantly greater than those of the original strain (t = 10.93; P = 1.00) and strain *sp1* (t = 8.12; P = 1.00). The lag phase of 7 h was similar to that for strain *sp1*. Following the addition of 0.5 μg of nitrapyrin ml^{-1} to stationary-phase cells there was no extension of the lag phase, but the specific growth rate was significantly reduced to 0.0342 h^{-1} (t = 6.15; P = 1.00), while the addition of nitrapyrin to exponentially growing cells resulted in an immediate reduction of the growth rate to 0.0185 h^{-1} (Fig. 4). Again, the reduction in the specific growth rate was greater

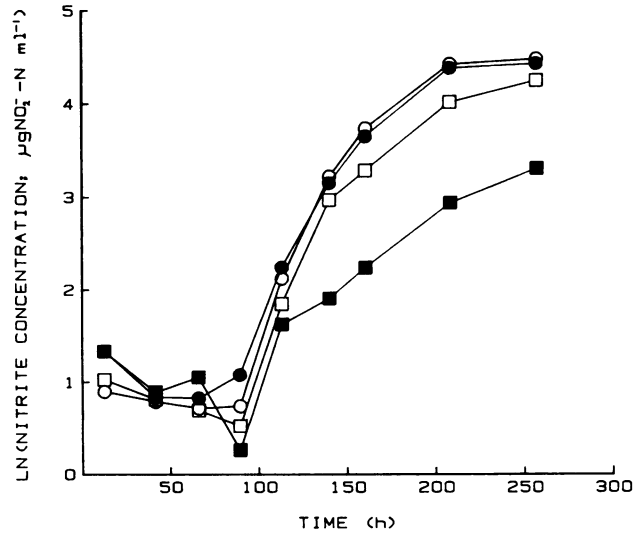

FIG. 5. Production of nitrite during growth of *N. europaea sp1* in soil following the addition of 0 (○), 1.25 (●), 3.75 (□), and 7.50 (■) μg of nitrapyrin ml^{-1} during exponential growth (125 h after inoculation). Data are presented from one flask of a set of three replicate flasks.

TABLE 3. Effect of nitrapyrin on the specific growth rate of *N. europaea sp1* growing exponentially in soil

Nitrapyrin concn (μg ml^{-1})	Specific growth rate (h^{-1})	Degrees of freedom	t^a	Probability
0.0	0.0419			
0.125	0.0404	4	0.27	0.20
0.25	0.0495	4	0.49	0.35
1.25	0.0358	4	1.04	0.64
3.75	0.0391	4	0.37	0.27
7.5	0.0121	4	8.16	1.00

[a] The Student t tests were carried out to compare specific growth rates with that of the control (0.0 μg ml^{-1}).

following addition to exponentially growing cultures (t = 3.79; P = 1.00). The corrected specific growth rate was 0.0301 h^{-1}, which is significantly less than that of the control (t = 9.63; P = 1.00) but greater than the apparent rate of 0.0195 h^{-1} (t = 3.5; P = 0.99). MPN data are not available for this strain, but the results suggest a significant bactericidal effect following the addition of nitrapyrin to exponentially growing cultures.

Hydrolysis of nitrapyrin. The rate of chemical hydrolysis of nitrapyrin to the less inhibitory 6-chloropicolinic acid (13) was measured for initial concentrations of 30, 50, and 70 μg of nitrapyrin ml^{-1} in medium described by Skinner and Walker (17). The rate of hydrolysis was independent of the initial concentration and followed first-order reaction kinetics with a rate of 0.0247 h^{-1} and a half-life of 27.9 ± 0.46 h (SE; n = 3). The nitrapyrin concentrations tested were greater than those used in experimental studies to facilitate assessment of concentrations, but it is assumed that the specific rate of hydrolysis is similar at lower concentrations. The effect of a reduction in the nitrapyrin concentration must therefore be considered in long incubations in which nitrapyrin is added to stationary-phase cultures.

Soil incubation studies. Ammonium oxidation in soil by *N. europaea sp1* proceeded to completion because of the buffering capacity of the soil which prevented inhibition by production of acid. Nitrite concentrations were also higher than in liquid culture because of the presence of ammonium in unamended soil, but there was no evidence of substrate inhibition. The nitrite concentration again increased exponentially, allowing the calculation of specific growth rates, as in the liquid culture. In the absence of nitrapyrin the specific growth rate of the original strain was 0.0419 h^{-1}, which is not significantly different from the rate in liquid culture (t = 0.86; P = 0.58), but the duration of the lag phase increased to 80 h.

Because of the likelihood of a significant reduction in the concentration of nitrapyrin as a result of hydrolysis during the long lag phases observed in soil culture, the addition of nitrapyrin was made to exponentially growing cultures only. The effect of the addition of several concentrations is illustrated in Fig. 5, and specific growth rates for all concentrations tested are summarized in Table 3. These data show that, of the concentrations tested, only 7.5 μg of nitrapyrin ml^{-1} (μg g^{-1} of soil) caused significant reduction in the specific growth rate (t = 8.16; P = 1.00).

DISCUSSION

Repeated subculturing of *N. europaea* in liquid batch culture led to the selection of strains with different growth

characteristics and differences in response to inhibition by nitrapyrin. The specific growth rates of both the original strain and strain *sp1* were comparable with previously reported values, but the specific growth rate of strain *sp2* was double that of the other two strains, suggesting a significant genotypic difference. The observed variation in growth characteristics emphasizes the need to monitor closely the growth characteristics of laboratory cultures, particularly when they are maintained by regular subculturing.

All three strains were inhibited by 0.5 μg of nitrapyrin ml^{-1} in liquid culture, and this value is within the range of previously reported inhibitory concentrations (1, 4, 8, 15, 16). Stationary-phase cells of each strain were less sensitive to nitrapyrin than exponentially growing cells. Following the addition of 0.5 μg of nitrapyrin ml^{-1} to the parent strain there was an extended lag of 34 h, which is longer than the calculated half-life of the inhibitor. During this lag period a significant proportion of nitrapyrin would have hydrolyzed to 6-chloropicolinic acid, which is a less effective inhibitor of autotrophic ammonia oxidation (13). Therefore, the reduced sensitivity of stationary-phase cells of *N. europaea*, as measured by the specific growth rate, may have been due to hydrolysis of nitrapyrin during the extended lag phase. However, stationary-phase cells of strains *sp1* and *sp2* were also less sensitive than exponentially growing cultures, yet no extension of the lag phase was observed. The difference in sensitivity of cultures at different stages of growth cannot, therefore, be attributed entirely to the chemical instability of the inhibitor. The reduced sensitivity of stationary-phase cells may result from a requirement for uptake of nitrapyrin before inhibition, which will be faster in exponentially growing than in stationary-phase cells. More information is required on the mode of action of this inhibitor to explain these differences.

Although the MPN method for the determination of viable cell concentration is widely used in studies of autotrophic nitrification, it has a high inherent statistical error (5). It does, however, provide qualitative information regarding bactericidal effects of nitrapyrin. The results shown in Table 2, combined with the use of a simple model to analyze kinetic data, suggest that the bactericidal activity of nitrapyrin varies significantly, depending on the strain used. The variation in specific growth rates and bactericidal effects among different strains highlights the importance of studying strain sensitivity and, in turn, the value of studies in which axenic cultures are employed. Similar differences in sensitivity of ammonia oxidizer strains were reported by Belser and Schmidt (1), but results of our studies show that such differences are not required to explain protection from inhibition by soil.

Comparisons were made between *N. europaea sp1* in liquid batch culture and in sterile soil. In the absence of nitrapyrin, specific growth rates were similar in soil and liquid culture, but the lag phase was extended by several days in the former. Losses of nitrapyrin as a result of hydrolysis would have been substantial during lag periods, and the inhibitor therefore was added to exponentially growing cultures only. *N. europaea sp1* was less sensitive to nitrapyrin in soil than in liquid culture, with an MIC 10 times greater than that in liquid culture. Soil therefore offered significant protection from inhibition. Underhill and Prosser (18) reported similar results when they tested the effect of potassium ethyl xanthate on *N. europaea* and *Nitrobacter* species.

Some of the observed difference in sensitivity to nitrapyrin may be explained by adsorption of the inhibitor to the soil organic fraction. It has been reported that as the concentration of organic matter increases, the effectiveness of nitrapyrin decreases (3, 6, 11). However, inhibition of autotrophic ammonia oxidation has been shown to vary between soil batches and soil types (6), and only a proportion of this variability can be directly accountable to organic matter and other physicochemical factors. Protection from inhibition may also result from the growth of nitrifiers at the surface of soil particles (10), which may create a microenvironment around the cells in which the inhibitor is less effective. Alternatively, growth at the surface of soil particles may spatially separate the bacteria and inhibitor. The protective effect of soil was significant, with a 10-fold greater concentration of nitrapyrin required for inhibition equivalent to that in liquid culture. Elucidation of the mechanisms involved in this protection is important for both optimization of inhibition by applied nitrapyrin and for the future development of new inhibitors. In addition, the significant differences between sensitivities of the stationary-phase and exponentially growing liquid cultures suggest that the timing of application of inhibitor may also be important and that actively nitrifying soils may be more sensitive than those to which ammonium has just been applied.

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