Effects of Pesticides on Nitrite Oxidation by Nitrobacter agilis

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The influence of pesticides on the growth of Nitrobacter agilis in aerated cultures and on the respiration of N. agilis cell suspensions and cell-free extracts was studied. Two pesticides, aldrin and simazine, were not inhibitory to growth of Nitrobacter, but five compounds [isopropyl N-(3-chlorophenyl) carbamate (CIPC), chlordane, 1,1-dichloro-2,2-bis (p-chlorophenyl) ethane (DDD), heptachlor, and lindane] prevented growth when added to the medium at a concentration of 10 μg/ml. Whereas CIPC and eptam prevented nitrite oxidation by cell suspensions, the addition of DDD and lindane resulted in only partial inhibition of the oxidation. Heptachlor and chlordane also caused only partial inhibition of oxidation, but were more toxic with cell-free extract nitrite oxidase. None of the pesticides inhibited the nitrate reductase activity of cell-free extracts, but most caused some repression of cytochrome c oxidase activity. Heptachlor was the most deleterious compound.

Biological nitrification, the oxidation of ammonium to nitrate, is largely caused by the autotrophic soil bacteria of the genera Nitrosomonas and Nitrobacter.

Various pesticides were tested for an effect on nitrification. Most of the studies used field trials or determinations in the soil perfusion unit developed by Lees and Quastel (12). Some (8, 11, 15) used both the perfusion apparatus and the manometric technique to measure the effect of pesticides on the nitrifiers. When respiration studies were made, soil which was percolated with an ammonium salt solution to increase the nitrifying population was usually added to the Warburg vessels. Seldom were pesticides tested for an effect on respiration with concentrated cell suspensions of either Nitrosomonas or Nitrobacter. Likewise, aerated liquid cultures of these organisms were infrequently used as a test system for pesticides.

The purpose of this study was to determine the mode of action of several pesticides on both growth in aerated cultures of Nitrobacter agilis and on the respiration of cell suspensions. Several enzymes were selected for study of pesticides which inhibited both growth and respiration.

MATERIALS AND METHODS

Pesticides. The following pesticides were tested: aldrin (1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-

hexahydro-1, 4-endo-exo 5,8 dimethanonaphthalene), chlordane (1,2,3,5,6,7,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene), CIPC [isopropyl N-(3-chlorophenyl) carbamate], DDD [1,1-dichloro-2,2-bis (p-chlorophenyl) ethane], eptam (S-ethyl-di-N,N-propyl-thiocarbamate), heptachlor (1,4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-endomethanoindene), lindane (gamma isomer of 1,2,3,4,5,6-hexachloro-cyclohexane), and simazine [2-chloro-4,6-bis (ethyl-amino)-s-triazine].

Growth. Nitrobacter agilis (ATCC 14123), kindly supplied by David Pramer, Rutgers University, New Brunswick, N.J., was grown in the clear inorganic medium of Aleem and Alexander (2), supplemented with NaMoO₄·2H₂O as suggested by Finstein and Delwiche (10).

To determine the pesticide effects, cultures had 10% nitrogen in medium 1970. The medium contained 100 ml of sterile medium in a 250-ml Erlenmeyer flask. With simazine only, 12.5 mg of the dry chemical was added to the culture flasks. Control flasks received 0.1 ml of acetone. All flasks were inoculated with cells in the log phase (1%, v/v) which had oxidized 80 μg of NO₃-N per ml. Flasks were incubated on a rotary shaker at 30°C. Every 12 hr the nitrite concentration was determined by the method of Rider and Mellon (17) as a measure of growth. The range of pesticide concentrations tested was 1 to 250 μg added per ml.

Large-scale cultivation. Cultures (400 ml) which oxidized 1 mg of nitrite nitrogen per ml were used as inocula for 10 liters of medium contained in a MF-14 Microferm fermentor (New Brunswick Scientific Co.) which was operated at 30°C with an aerator rate of 6 liters of air per min. After oxidation of 1.5 mg of nitrite nitrogen per ml, cells were har-
vested by continuous-flow centrifugation (Sorvall model SS-1 centrifuge equipped with the Szent-
Gyorgyi and Blum continuous-flow system). The cells were washed three times with cold distilled water and then suspended in 0.1 M tris(hydroxymethyl)-
aminomethane (Tris)-hydrochloride buffer (pH 8.0) at a concentration of 1 g (wet weight) per ml.

Preparation of cell-free extracts. Cell paste (5 g) suspended in 5 ml of the sonication solution of Aleem (1) was disrupted at 5 C for 15-sec intervals for a total period of 3 min with a 100-w ultrasonic dis-
integrator (Measuring & Scientific Equipment, Ltd., London, England). The broken cell suspension was centrifuged at 40,000 X g for 10 min at 4 C in a refrigerated centrifuge (model RC-2; Ivan Sorvall, Inc., Norwalk, Conn.), and the supernatant fluid was stored at 4 C.

Purity of cultures. All cultures were routinely checked for heterotrophic contaminants by inoculating nutrient broth in quadruplicate and incubating one pair at 30 C and the other at 37 C for 7 days. In addition, each of the large-scale cultures was tested for purity by streaking samples on (i) nutrient agar, (ii) nutrient agar with 1% yeast extract, (iii) Czapek Dox solution agar, (iv) basal salt medium with agar and 0.5% yeast extract, (v) basal salts with nutrient broth (0.1%) and glucose (0.2%). Duplicate plates were incubated for 7 days at 30 C, and another set was incubated at 37 C. Whenever contamination was detected, the cultures were discarded.

Oxidation of nitrite. The effect of pesticides on nitrite oxidation was determined in the Warburg respirometer. The cell suspensions and cell-free extracts were diluted so that 50 to 60 ml of O2 were consumed per hour. Pesticides, dissolved in acetone, were added to the Warburg vessels in 10-
mliter volumes or less. Although acetone did not affect the oxidation, controls containing the same volume of acetone were included. Initial activities were com-
pared in the presence and absence of pesticides. At the end of 1 hr, samples were removed from the Warburg flasks and assayed for nitrite concentration by the method of Rider and Mellon (17).

Nitrate reductase. The procedure of Straat and Nason (21) was modified for determining the nitrate reductase activity. In a total of 1.0 ml, the reaction mixture contained 0.5 ml of 0.1 M acetate buffer (pH 6.0), 0.1 ml of 0.1 M KNO3, 0.1 ml of suitably diluted cell-free extracts, 0.15 ml of 1% (v/v) mammalian cytochrome c (Sigma Chemical Co., St. Louis, Mo.), and 0.25 ml of 2 X 10-4 M sodium ascorbate. The reaction, maintained at 30 C, was started by addition of KNO3; the nitrite formed was measured at the end of 10 min.

Reduced nicotinamide adenine dinucleotide (NADH) oxidase. NADH oxidase was measured by the method of Smith and Hoare (19). The decrease in absorbance of NADH at 340 nm produced by the cell-free extract was measured at 30-sec intervals for a 10-min period with a Beckman model DU spectrophotometer.

Cytochrome c oxidase. To measure cytochrome c oxidase activity, the following reaction system was used: 0.1 ml of 1.0 M Tris buffer (pH 8.0), 0.1 ml cell-free extract (0.36 mg of protein), 0.3 ml of 1% (w/v) cytochrome c, 0.3 ml of 4 X 10-4 M sodium ascorbate, and 0.3 ml of deionized, distilled water. The reaction was started by addition of cell-free extract, and the decrease in absorbance was measured at 550 nm with a Gilford-2000 multiple sample ab-
sorbance recorder attached to a Beckman model DU spectrophotometer. Initial activities were corrected by subtracting a “blank” representing the decrease in absorbance during absence of enzyme. Because the pesticides were added in acetone, an equivalent amount of acetone was added to the control cuvettes.

Protein. Protein concentrations were determined by the method of Lowry et al. (14). Crystallized, bovine fraction V albumin (Sigma Chemical Co.) was used as protein standard.

RESULTS

Growth effects. The effects of the pesticides on growth of N. agilis are illustrated in Table 1. Only two compounds, aldrin and simazine, were

| Pesticide | Conc. | NO3-N remaining after indicated days | Range of inhibition |
|-----------|------|-----------------------------------|--------------------|
| Aldrin    | 250  | 42 20 0 0                          | 0                  |
| 10        | 42   | 42 42 42 22                        | 0                  |
| CIPC      | 25   | 42 42 42 42                        | 0                  |
| 10        | 22   | 18 0 0                             | 0                  |
| DDD       | 10   | 42 42 42 42                        | 5                  |
| 5         | 42   | 22 0 0                             | 0                  |
| Eptam     | 75   | 42 42 42 42                        | 25-75              |
| 50        | 42   | 42 31 20                           | 1                  |
| 25        | 42   | 42 17 17                           | 0                  |
| Heptachlor| 25   | 42 42 42 42                        | 10                 |
| 10        | 42   | 42 42 42                           | 0                  |
| 5         | 42   | 32 22 0                            | 0                  |
| Lindane   | 10   | 42 42 42 42                        | 10                 |
| 5         | 42   | 42 14 1                            | 0                  |
| Simazine  | 250  | 32 10 0 0                          | 0                  |
| Control   | 25   | 0 0 0 0                            | 0                  |

* Initial nitrite concentration in all flasks was 42 µg of NO3^-N per ml. All values are expressed as micrograms per milliliter.

* This was the amount added per milliliter of medium. In most cases it was above the solubility level of the individual pesticide.
not inhibitory under the conditions of the experiment. The other six compounds: lindane, DDD, chlordane, heptachlor, CIPC, and eptam prevented nitrite oxidation for 9 days, whereas control flasks exhausted the nitrite within 5 days. Based on the concentration of pesticide required for 100% inhibition, the compounds were arranged in the following order: DDD (5 µg/ml), chlordane (5 µg/ml), CIPC (10 µg/ml), heptachlor (10 µg/ml), lindane (10 µg/ml), and eptam (75 µg/ml).

Oxidation studies with cell suspensions and cell-free extracts. To determine whether the growth inhibitors acted on nitrite oxidase of *Nitrobacter*, nitrite oxidation of cell suspensions was measured by oxygen consumption and nitrite disappearance in Warburg respirometer vessels. Of the six pesticides which inhibited growth, only CIPC and eptam appreciably affected the nitrite oxidase activity of cell suspensions (Table 2). However, 110 µg of CIPC per ml and 275 µg of eptam per ml were required for total inhibition of oxidation,

|TABLE 2. Effect of pesticides on nitrite oxidation by cell suspensions and cell-free extracts of Nitrobacter agilis |
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|**Whole cell suspensions**

| Pesticide | Amplitude added | Oxygen uptake | Nitrite consumed | Pesticide | Amplitude added | Oxygen uptake | Nitrite consumed |
|---|---|---|---|---|---|---|---|
| None | µg/ml | µliters | % | µmoles | % | None | µg/ml | µliters | % | µmoles | % |
| CIPC | 110 | 48.0 | 100.0 | 4.57 | 100.0 | CIPC | 125 | 44.2 | 100.0 | 4.1 | 100.0 |
| | 100 | 8.5 | 17.8 | 0.23 | 5.0 | | 50 | 36.9 | 83.5 | 3.65 | 89.0 |
| | 90 | 8.0 | 16.7 | 0.51 | 11.1 | | | | | |
| | 75 | 12.8 | 26.6 | 0.83 | 18.0 | | | | | |
| | 50 | 25.4 | 53.0 | 2.43 | 53.0 | | | | | |
| Chlordane | 500 | 9.6 | 20.0 | 1.14 | 25.0 | Chlordane | 250 | 0.0 | 0.0 | 0.3 | 7.3 |
| | 250 | 15.8 | 33.0 | 2.1 | 46.0 | | 200 | 5.4 | 11.5 | 0.4 | 9.7 |
| | 125 | 9.6 | 18.4 | 3.02 | 66.0 | | 150 | 24.3 | 55.0 | 1.8 | 44.0 |
| DDD | 500 | 11.5 | 24.0 | 1.59 | 34.7 | DDD | 500 | 20.0 | 46.0 | 2.29 | 56.0 |
| | 375 | 17.6 | 36.6 | 1.44 | 31.4 | | | | | |
| | 250 | 20.1 | 41.7 | 1.8 | 39.4 | | | | | |
| | 125 | 33.8 | 70.0 | 2.66 | 58.0 | | | | | |
| Eptam | 275 | 0.0 | 0.0 | 0.0 | 0.0 | Eptam | 275 | 0.0 | 0.0 | 0.5 | 12.2 |
| | 250 | 9.6 | 2.0 | 0.57 | 12.5 | | 250 | 12.1 | 27.4 | 0.47 | 11.5 |
| | 225 | 9.0 | 18.7 | 0.53 | 11.5 | | 150 | 31.0 | 70.2 | 2.71 | 66.0 |
| | 200 | 10.2 | 21.2 | 1.37 | 30.0 | | | | | |
| | 150 | 20.6 | 43.0 | 2.17 | 47.0 | | | | | |
| Heptachlor | 500 | 21.0 | 43.7 | 2.47 | 54.0 | Heptachlor | 250 | 0.0 | 0.0 | 0.1 | 2.4 |
| | 250 | 30.0 | 62.5 | 3.0 | 65.5 | | 200 | 0.0 | 0.0 | 0.3 | 7.3 |
| | 125 | 32.2 | 67.0 | 3.8 | 83.0 | | 150 | 18.8 | 42.5 | 1.11 | 27.0 |
| Lindane | 500 | 22.4 | 46.5 | 1.78 | 39.0 | Lindane | 500 | 33.6 | 76.0 | 3.4 | 83.0 |
| | 250 | 48.0 | 100.0 | 4.65 | 102.0 | | | | | |

Oxidation studies with cell suspensions and cell-free extracts. To determine whether the growth inhibitors acted on nitrite oxidase of *Nitrobacter*, nitrite oxidation of cell suspensions was measured by oxygen consumption and nitrite disappearance in Warburg respirometer vessels. Of the six pesticides which inhibited growth, only CIPC and eptam appreciably affected the nitrite oxidase activity of cell suspensions (Table 2). However, 110 µg of CIPC per ml and 275 µg of eptam per ml were required for total inhibition of oxidation,

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*Oxidation rate, expressed as µliters of O₂ and as µmoles of nitrite consumed per milligram (dry weight) of cells per hour.*

*Oxidation rate, expressed as µliters of O₂ and as µmoles of nitrite consumed per milligram protein per hour.*

*Each vessel contained 0.1 ml of either cells (1.21 mg, dry weight) or cell-free extract (1.06 mg of protein). 0.5 ml of 2.0 × 10⁻⁴ M NaNO₃ (10 µmoles), 0.3 ml of 1 M Tris buffer (pH 8.0), and 1.9 ml of distilled water. The pesticides were added in 10 µliters of acetone. Controls received 10 µliters of acetone, and endogenous rates were determined in the presence of acetone (10 µliters) and also with the pesticides.*

*Simazine was added to Warburg vessels in 2 ml of methanol and evaporated to dryness at 50°C. Control vessels received an equivalent volume of methanol.*

*Q₂₀ is expressed as microliters of O₂ consumed per milligram (dry weight) of cells per hour, or as microliters of O₂ consumed per milligram of protein per hour (cell-free extracts).*
but only 10 µg of both pesticides per ml were required for cessation of growth (Table 1).

The nitrite oxidase activity of cell-free extracts was then tested in the presence of pesticides. Eptam, CIPC, DDD, and lindane affected both cell suspension and cell-free extract activities similarly (Table 2), but heptachlor and chlordane were more inhibitory for the nitrite oxidase in the cell-free extracts. The extract activity was completely inhibited by the addition of 250 µg of chlordane and 200 µg of heptachlor per ml, but the nitrite oxidase of cell suspensions was restrained by only 50 and 75 to 80% with 500 µg of heptachlor and chlordane, respectively. Neither DDD nor lindane at a concentration of 500 µg/ml caused cessation of nitrite oxidation by either cell suspension or cell-free extracts.

Nitrite oxidase as measured by oxygen consumption differed slightly from the nitrite disappearance at a given pesticide concentration because oxygen uptake was reported at initial rate, but the nitrite concentration was determined at the end of 1 hr. In some instances, the initial oxidation rate was zero, but a small amount of oxygen was consumed before the end of the 1-hr assay period.

**Kinetics of oxidation.** By using cell suspensions, three pesticides (CIPC, 75 µg/ml; eptam, 200 µg/ml; and DDD, 600 µg/ml) were selected for further study in the respirometer. The concentrations of nitrite added to the Warburg flasks varied from 1.5 to 12 µmoles. The types of inhibition which occurred (Fig. 1) were noncompetitive, competitive, and uncompetitive with CIPC, eptam, and DDD, respectively. The Michaelis constant (K_m) for the nitrite oxidation without pesticides was 3.76 mm, whereas those for the inhibited reactions were: CIPC, 3.76 mm; eptam, 5.49 mm; and DDD, 1.68 mm.

**Nitrate reductase.** Nitrate reductase activity was measured with each of the pesticides which affected nitrite oxidase, but no deleterious effect was observed by any of the compounds in concentrations up to 500 µg/ml (data not tabulated). A representative value for the nitrite produced by the cell-free extract during the 10-min reaction was 0.025 µmoles per mg of protein.

**NADH_2 oxidase.** When the NADH_2 oxidase activity of cell-free extracts was tested at 340 nm in a reaction mixture containing 0.1 ml of 2 × 10^{-4} M NADH_2, 0.1 ml of 0.5 M MgCl2, 0.1 ml of 1.0 M Tris (pH 8.0), 0.1 ml cell-free extract (0.365 mg of protein), and water to 1.0 ml, a decrease in absorbance of 0.18 per mg of protein was noted at 10 min. This activity was not affected by the addition of 10 µmoles of azide. A slightly increased rate (Δ optical density of 0.25 per mg of protein) was obtained by additions of either flavine mononucleotide or flavine adenine dinucleotide at 2 × 10^{-4} M final concentration, but control cuvettes with azide (10 µmoles) had equivalent rates. Likewise, using a phosphate buffer (0.05 M) at pH 7 and 8 in the presence and absence of MgCl2 (0.05 M) resulted in no apparent NADH_2 oxidase activity.

**Cytochrome c oxidase.** Pesticides which affected the nitrite oxidase activity of cell-free extracts

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**FIG. 1.** Nature of the inhibition of nitrate oxidation by three different pesticides. Each Warburg flask contained 0.1 ml of *Nitrobacter agilis* cell suspensions (1.3 mg, dry weight), 0.5 ml of NaNO3 (2 to 12 µmoles), and 0.3 ml of Tris buffer (pH 8.0). The center well contained 0.2 ml of 20% (w/v) KOH.
were tested for an effect on cytochrome c oxidase. Most of the pesticides tested inhibited cytochrome c oxidase to some degree, but none suppressed the activity completely (Table 3). Heptachlor, the most toxic, inhibited the activity by 76% at a concentration of 500 μg/ml, but completely suppressed nitrite oxidase at 200 μg/ml (Table 2). At 500 μg/ml, DDD caused a similar effect on both oxidases, as relative activities of 56 and 66% were obtained for nitrite oxidase and cytochrome c oxidase, respectively. Eptam was unique in this series of compounds, as a slight, but reproducible, stimulation of cytochrome c oxidase was noted.

**DISCUSSION**

The eight pesticides considered in this report are currently recommended for agricultural uses. Field studies or laboratory investigations with soil systems have indicated no inhibition with aldrin (4, 9, 18), simazine (4), heptachlor (18), or DDD (4). The two chemicals (CIPC and eptam) which inhibited both the growth of *Nitrobacter* and nitrite oxidation were also inhibitory to nitrification in field studies (4, 7, 16). With applications of 1,000 lb per acre, Brown (5) observed retardation of nitrification with lindane and chlordane, but Shaw and Robinson (18) observed no effect with chlordane at 300 lb per acre.

We were particularly concerned in determining whether a correlation existed between cell suspension oxidation studies and the growth experiments. Aldrin and simazine neither affected growth in aerated cultures nor nitrite oxidation by cell suspensions or cell-free extracts. Although all six of the other pesticides tested caused cessation of growth with eptam being the least toxic (75 μg/ml were required for complete inhibition), only CIPC and eptam were completely toxic to the nitrite oxidase of cell suspensions. Although various degrees of nitrite inhibition were observed with chlordane, DDD, heptachlor, and lindane at 500 μg/ml, inhibition of nitrite oxidase activity is probably not the mode of action of these chemicals on *Nitrobacter* since 5 to 10 μg/ml stopped growth. Even with CIPC and eptam, inhibition of nitrite oxidase might not be the main mode of action since the concentrations of CIPC and eptam required for cessation of nitrite oxidase were approximately 10- and 4-fold greater, respectively, than the concentrations necessary for inhibiting growth.

It is noteworthy that heptachlor and chlordane were more inhibitory with cell-free extract oxidation than with cell suspension oxidation. Complete inhibition of the nitrite oxidase activity in cell-free extracts occurred with additions of 250 μg of chlordane per ml and 200 μg of heptachlor per ml, but additions of 500 μg/ml to cell suspensions resulted in 80% inhibition with chlordane and 50% inhibition with heptachlor. This difference in inhibition could be the result of an impermeability of the cell suspensions to these two compounds or to binding of protein in the cell-free extracts, since both chemicals are insoluble at the concentrations used. Likewise, sorption of cells or protein by insoluble pesticide particles could be responsible for the linear effect noted in the respiration experiments with increasing concentrations of the pesticides even though the solubility levels of the chemicals were exceeded.

Since marked inhibition of nitrite oxidase in cell-free extracts occurred with CIPC, chlordane, eptam, and heptachlor, the pesticides were tested for an effect on the cytochrome c oxidase activity in the cell-free extracts. Aleem and Nason (3) and Lees and Simpson (13) indicated that nitrite oxidation in *Nitrobacter* is mediated by cytochrome of c-like and a-like components, but Aleem (1) recently showed that NO$_3^-$ oxidation is mediated by cytochrome $a$, and cytochrome oxidase components with the energy dependent reduction of cytochrome $c$. None of the pesticides examined by us inhibited the cytochrome $c$ oxidase activity sufficiently to account for the nitrite oxidase inhibition observed with cell suspensions and cell-free extracts. The per cent of relative cytochrome $c$ oxidase activity obtained with 500 μg of heptachlor per ml was similar to cell suspension respiration results, but heptachlor caused total inhibition of cell-free extract nitrite oxidase at 200 μg/ml. Thus, effects observed with heptachlor were probably only partially due to cytochrome $c$ oxidase inhibition.

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**TABLE 3. Effect of pesticides on cytochrome c oxidase**

| Compound   | Final concentration μg/ml | Activity $a$ | Relative activity % |
|------------|----------------------------|--------------|---------------------|
| Control    |                            | 0.139        | 100                 |
| Chlordane  | 500                        | 0.081        | 58                  |
| CIPC       | 500                        | 0.069        | 50                  |
| DDD        | 500                        | 0.092        | 66                  |
| Eptam      | 500                        | 0.167        | 120                 |
| Heptachlor | 500                        | 0.033        | 24                  |

*a Reaction mixture contained 0.1 ml of 1.0 M Tris buffer (pH 8.0), 0.1 ml of cell-free extract (0.36 mg of protein), 0.3 ml of 1% (w/v) cytochrome c, 0.3 ml of 2 × 10$^{-4}$ M sodium ascorbate, and 0.3 ml of distilled water. Sodium cyanide (10 μmole) completely inhibited the reaction.

$b$ Activity expressed as decrease in absorbance at 550 nm per min per mg of protein.
Although it is probable that NADH oxidase is used for autotrophic growth only by the hydrogen bacteria (20), Aleem (1) showed that NADH oxidation in Nitrobacter proceeds by an electron transport chain analogous to the mitochondrial respiratory chain (see reference 6). Consequently, we were interested in studying the effects of pesticides on the oxidase as an indication of cytochrome inhibition. However, we were unable to detect NADH oxidase activity in our preparations.

Straat and Nason (21) postulated that the enzymatic reduction of nitrate in Nitrobacter might be the first step of a sequence for providing nutritional nitrogen, or it could be a means of recycling nitrite for nitrite oxidation. No deleterious effect on nitrate reductase was noted in our system upon addition of the pesticides.

Though we were unable to characterize the inhibitions caused by the pesticides studied, our results indicate that growth studies with low concentrations of pesticides are more suitable than the measurement of nitrite oxidation as indexes of the toxicity of pesticides for Nitrobacter agilis. With growth studies, inhibition of biosynthetic reactions will be detected, whereas only inhibition of energy assimilation is detected by nitrite oxidation studies.

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