The Effect of 2,3,6-Trichlorophenylacetic and 2,2-Dichloropropionic Acids\(^1\) on Nitrite Oxidation

JERRY V. MATEUX AND ARTHUR R. COLMER

Department of Bacteriology and Agricultural Experiment Station, Louisiana State University, Baton Rouge, Louisiana

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ABSTRACT

MATEUX, JERRY V. (Louisiana State University, Baton Rouge), AND ARTHUR R. COLMER. The effect of 2,3,6-trichlorophenylacetic and 2,2-dichloropropionic acids on nitrite oxidation. Appl. Microbiol. 10:206–210. 1962. — The effects of two herbicides, 2,3,6-trichlorophenylacetic acid, sodium salt, and 2,2-dichloropropionic acid, sodium salt, on nitrite-oxidizing bacteria were studied by the soil perfusion technique. The time of application of 2,3,6-trichlorophenylacetic acid affected its toxicity to the nitrifier. When it was present in the environment as the nitrifier started growth, it was more toxic than if the organisms were allowed to nitrify actively before they were subjected to the herbicide.

The herbicide 2,2-dichloropropionic acid at rates up to 700 ppm had little effect on nitrite oxidation. The toxicity of 2,3,6-trichlorophenylacetic acid for \textit{Nitrobacter} was reduced by 2,2-dichloropropionic acid irrespective of whether the cells came into contact with the agents before or during active oxidation. The mode of action for this phenomenon has not been determined.

Nitrification, that portion of the nitrogen cycle involving the conversion of the ammonium ion to the nitrate ion, is a two-step process brought about by organisms specific for each phase. The end products of these oxidations are very important in agriculture; the presence of excess ammonium or nitrite ion is deleterious to the growth of many agricultural crops, and nitrate serves as the main nitrogen source for most plants. It follows then that any soil additive which may interfere with either step of nitrification may thus be detrimental to the economy of the soil.

Herbicides, one group of soil additives, are in use in large quantities for weed control in agricultural croplands. Some herbicides are so effective as "soil sterilants" for plant growth that the question arises as to their possible effect on the soil microflora. This report describes the results of studies of two such herbicides on the nitrite oxidation phase of nitrification.

\(^1\) The trade name for 2,3,6-trichlorophenylacetic acid, sodium salt, a product of AMCHEM, Ambler, Pa., is Fenac. The trade name for 2,2-dichloropropionic acid, sodium salt, a product of Dow Chemical Company, Midland, Mich., is Dalapon.

MATERIALS AND METHODS

Organism and culture medium. A pure culture of \textit{Nitrobacter} was obtained from liquid enrichments in Stephenson (1949) sodium nitrite medium modified by use of 1 g of NaNO\(_2\) and the omission of CaCl\(_2\). After many failures to secure pure cultures, a culture enriched with \textit{Nitrobacter} was centrifuged at 1,000 \(\times\) g for 30 min, and dilutions to extinction were made of the centrifuged supernatant, the sediment, and a portion of the culture that had not been centrifuged. These dilutions were made by adding 1 ml of the culture to 9 ml of mineral salts broth (MS) as the first dilution. Subsequent dilutions were made by removing 1 ml and transferring 0.5 ml to a 4.5-ml tube of MS broth and the remaining 0.5 ml to a tube of nutrient broth. The dilutions in nutrient broth were to determine the highest dilution of the MS broth in which the heterotrophic contaminants had been transferred. Any dilution of MS broth that nitrified beyond a dilution that showed growth in nutrient broth was further checked for heterotrophic contaminants by inoculating nutrient agar, Tryptone glucose extract agar, gelatin agar, starch agar, hydrogen sulfide agar, glycerol broth, and thioglycollate broth containing either glucose, sucrose, or glycerol (Difco, 1953). From the five enrichments exposed to such treatment, four pure cultures were obtained: one from the centrifuged sediment and three from the supernatant. In no attempt was a pure culture obtained from the uncentrifuged enrichment cultures.

Procedure for studying effect of herbicides. The effect of the herbicides on nitrification was studied by the soil perfusion technique (Audus, 1946; Lees and Quastel, 1946). The apparatus used was the Temple (1951) modification of the Lees unit.

Inocula preparation. The medium used in the perfusion units was the same as that used for growing the culture. Inocula for the perfusion units were grown on a rotary shaker and used when the nitrite supplied was oxidized completely to nitrate.

Soil preparation. Fresh Mhoon silt loam was air dried and sieved. We found that the 2- or 3-mm size crumbs were best suited for perfusion work. These soil crumbs were dispensed into test tubes in 10-g amounts and autoclaved for 2 hr at 121°C and were later used to fill the substrate tube of the perfusion unit. The autoclaving process aided...
in maintaining the integrity of the soil particles and thus minimized erosion during the perfusion process.

Assembly of unit components. That the substrate concentration would be the same in all flasks of an experiment, the same volume of medium, usually less than 200 ml, from the same lot was added to tubulated flasks. The flasks covered with aluminum foil and with a cotton plug in the air-intake tube were sterilized at 121 C for 15 min. After they had cooled to room temperature, the desired amount of the herbicides (sterilized by filtering a water solution of the herbicide through a Millipore filter) was added to duplicate flasks (comprising a set) along with sufficient sterile water to bring the total volume in the flasks to 200 ml.

A small plug of cheese cloth was placed in the bottom of the substrate tube of the perfusion unit to hold the column of soil in place. The outlet to the vacuum was covered with an aluminum foil cap and a rubber stopper was placed over the substrate tube. The unit was wrapped in Kraft paper and sterilized at 121 C for 15 min.

After cooling, the unit was positioned in the tubulated flask and approximately 10 g of the sterile soil crumbs were added to the substrate tube. Four milliliters of inoculum were added to the soil in 1-ml amounts over a 12-hr period to allow for maximal adsorption of organisms to the soil particles.

The units were set up in batteries connected to a vacuum source. The air-intake tubes of the flasks were connected to a water bottle so arranged that entering air was first bubbled through water to minimize evaporation of the medium in the units.

The effect of the herbicides on nitrite oxidation was determined by measuring the course of nitrite disappearance. Nitrite was determined by the method of Saltzman (1954) modified for use with aqueous solutions. The Klett-Summerson colorimeter was used with a 540 m filter for the determinations of nitrite.

RESULTS AND DISCUSSION

Effect on nitrite oxidation when herbicide was present at time of inoculation of perfusion units. The nitrite-oxidizing bacteria were not affected by the presence of Fenac below 100 ppm and only slightly inhibited by 200 ppm (Fig. 1). When 300 ppm were used, the oxidizing time was increased by approximately 10 days over the control; with either 400 or 500 ppm Fenac there was no indication of nitrification during the 14 days of perfusion.

Effect on nitrite oxidation when herbicide was added to perfusion units that were nitrifying linearly. In the original experiments the Fenac was present before the organisms started active growth. It was desirable to learn if the effects of the Fenac would be the same if it were added to cells actively nitrifying and, too, if there was any residual effect of the agent on the organisms.

Two sets of units were prepared and perfused to completion. Figure 2 indicates that this was accomplished in about 4 days. At the point of completion, the old medium was removed and fresh nitrite medium added to the two sets. To one set 300 ppm Fenac were added, whereas the other set was left as a control.

Figure 2 shows that these two sets nitrified at approximately the same rate, a finding differing from the results shown in Fig. 1 where the 300 ppm Fenac added initially delayed the completion of nitrite oxidation. The reason for this lessened toxicity of the Fenac for actively nitrifying cells as contrasted to the evident toxicity of the agent at the beginning of the test is not known. Quastel and Scholefield (1951) found in their perfusion studies of soil saturated with nitrifiers “that a condition of bacterial saturation of the surfaces of the soil crumbs arises when, in effect, the system behaves like a suspension of nonproliferating and yet actively metabolizing cells.”

Possibly the difference in resistance of the nitrifiers to Fenac under our two conditions is related to their proliferation. When the inoculum was first placed on crumbs of soil, the soil surfaces were not saturated with cells, and the cells with fresh medium available started active growth. It was these actively proliferating cells which were more susceptible to the inhibitory effect of Fenac than were the mature nonproliferating cells.

Since no enumeration of cells was made on the perfusion units when inoculated nor on the units at the point of emptying the old medium, no correlation can be made between toxicity and relative numbers of cells as the explanation for the observed results. Quastel and Scholefield pointed out the difficulties attendant upon attempts to study numbers of cells found in their perfusion units.

The upper curve of Fig. 2 shows that there was no residual toxic effect of 300 ppm Fenac on Nitrobacter. With 300 ppm present initially, 14 days were required for the complete oxidation of the nitrite as contrasted to

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* Millipore Filter Corporation, Bedford, Mass.
* Klett Manufacturing Company, New York, N. Y.

**FIG. 1.** Effect of Fenac concentration on nitrite oxidation
approximately 4 days of the control. However, when the old medium containing Fenac was removed and fresh medium without Fenac was perfused over the soil column, the organisms responded as though they had not been previously exposed to the toxic agent, i.e., they oxidized the nitrite at the same rate as the original control.

Other studies (Mayeux, 1961) with *Nitrobacter* showed that when actively nitrifying cells were treated with 600 ppm Fenac, they appeared to start oxidation, but ceased to nitrify between 12 and 24 hr after perfusion had been started. An additional 6 days of perfusion failed to show any oxidation by the cells. Even when the Fenac was removed and fresh medium was added, there was no recovery of the organisms. This lethality of 600 ppm was also demonstrated by adding this level to cells which had grown in the presence of 300 ppm. Here again active nitrification was stopped by the 600 ppm concentration. The fact that the organisms had overcome the first en-

![Graph](http://aem.asm.org/)

**FIG. 2.** Nitrite oxidation in perfused soil without and then with 300 ppm Fenac, contrasted with that in soil which initially contained Fenac and was then washed free of the herbicide.

![Graph](http://aem.asm.org/)

**FIG. 3.** Effect of Dalapon on inhibition of nitrite oxidation by Fenac. Herbicides present at time of inoculation of perfusion unit.
counter with the agent did not assure that they would be more tolerant of higher concentrations.

Effect on nitrification when two herbicides were used together. Perfusion studies with Dalapon\(^1\) showed that rates up to 700 ppm had little effect on nitrite oxidation (Mayeux, 1961). In these studies the Dalapon was added initially. E. R. Stamper (personal communication, 1960) found, in tests with mixtures of Fenac and Dalapon for destroying unwanted plants in sugar cane fields, that the two herbicides used together had no additive effect; indeed, the presence of Dalapon in the mixture with Fenac did not increase this destruction beyond that of Dalapon when used alone. He also found that the use of the Fenac-Dalapon mixture caused a decrease in the residual preemergent effect of the Fenac on weed seeds.

Figures 3 and 4 give the results of tests to correlate Stamper's findings on plants with our results with *Nitrobacter*. Figure 3 shows that the control and 700 ppm Dalapon units nitrified at approximately the same rate, and the units with 300 ppm Fenac had their typical delay in completion, characteristic features when the agents were present initially. When 700 ppm Dalapon was added to 300 ppm Fenac, the toxicity was lessened and the cells oxidized the nitrite in approximately 10 days, about 3 days earlier than with 300 ppm Fenac alone.

Since in earlier work we found that organisms in the active stage of nitrification required a higher concentration of Fenac to exhibit toxicity, tests were devised to determine if Dalapon would reduce Fenac's toxicity under such a condition. Figure 4 gives the results of such a comparison. Dalapon (700 ppm) and 0, 300, 450, and 600 ppm Fenac were added to units which had been undergoing active nitrification. In all cases the units containing the combination of Fenac and Dalapon nitrified at a rate faster than did the sets with Fenac alone.

The explanation for this effect of Dalapon on Fenac in either the case of plants or *Nitrobacter* is not known. It does not seem likely that the Dalapon is providing a nutritive source to *Nitrobacter*, due both to its mode of nutrition and to the observed result that the Dalapon control unit did not nitrify at a rate as rapid as the culture control. The possibility of a chemical reaction between the two chlorinated compounds does not seem likely, but the possibility of reaction(s) with the other materials present in the commercial formulations is not ruled out.

Fenac as used in sugar cane fields has as "active ingredients" 16.1% of 2,3,6-trichlorophenylacetic acid, sodium salt, and 83.9% "inert ingredients." We secured a sample of the portion of active ingredients of Fenac free of the inert portion of the formulation. The manufacturer indicated that the sample was a mixture of isomers of 2,3,6-trichlorophenylacetic acid. Preliminary tests made with solutions of either the Fenac isomers or Fenac and Dalapon gave a white precipitate at the concentrations used. The nature of the precipitate has not been determined.

The literature on the nitrifiers confirms the effective use of perfusion units. In a recent report, Whiteside and Alexander (1960) stated that the Warburg manometric technique could be used to determine the effect of herbicides on the respiration of the soil microflora; Quastel and Scholefield (1951) reported that by using soil enriched with nitrifiers the effect of herbicides on their respiration could be determined. We have made only preliminary tests on the toxicity of Fenac for *Nitrobacter* when the cells were used on soil crumbs in the Warburg apparatus. In one 5-hr test with two levels, 400 and 500 ppm, we found the microliters of O\(_2\) uptake per hr varied but little between the control and test flasks.

Our limited data obtained in a direct comparison of the two techniques suggests a possible disadvantage of the Warburg procedure. In the perfusion studies (Fig. 4) it is obvious that the Fenac has to remain in contact with the organisms for several hours before inhibitory effect is noted, and that nitrification is not seriously retarded until after 12 hr of perfusion with 450 or even 600 ppm Fenac. The shorter times usually employed in the Warburg might not show these effects.

We wish to emphasize that although Fenac does inhibit nitrification, the levels necessary for this inhibition are far above the normal 1.5 to 3 lb (0.75 to 1.5 ppm) per acre used for weed control.

**LITERATURE CITED**


