

Nitrite and Nitrous Oxide Accumulation During Denitrification in the Presence of Pesticide Derivatives†

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Temporary accumulation of nitrite and nitrous oxide was observed in soil incubated under anaerobic conditions when derivatives of the insecticide chlordimeform [(*N*-4-chloro-*o*-tolyl)-*N,N'*-dimethylformamidine] were added. Chlordimeform did not affect the denitrification process, but *N*-formyl-4-chloro-*o*-toluidine and 4-chloro-*o*-toluidine caused an inhibition as determined by the accumulation of nitrite and nitrous oxide. A simultaneous application of the insecticide and its derivatives resulted in a stronger inhibitory effect than the application of each compound separately. Aniline intermediates of other pesticides also inhibited denitrification in soil, and they proved to be more effective than their parent compound.

The end product in denitrification is usually molecular nitrogen. However, under certain conditions and especially in the presence of certain chemicals exhibiting an inhibitory influence, the increased and prolonged accumulation of N₂O (nitrous oxide) and nitrite has been found (4, 5, 10). Whereas N₂ is an inert and harmless product, the other two nitrogen compounds are a source of concern in our environment. Nitrites may react with various chemicals to form toxic substances, and nitrites alone have been found to be toxic to plants, animals, and humans. Nitrous oxide is of concern because its release from soil to the atmosphere might cause damage of the stratospheric ozone layer.

Most pesticides applied at the recommended rates show little or no effect on the activities of soil bacteria (2). However, degradation products may be formed whose impact on certain physiological processes of non-target microorganisms can be more important than the original pesticide.

It has been demonstrated in soil perfusion experiments that linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea] and diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] added to soil at 100 ppm (wt/wt) inhibit nitrification with only trace amounts of NO₂⁻-N appearing. However, the addition of 3-(3,4-dichlorophenyl)-1-methylurea, the demethylation product of diuron or demethoxylation product of linuron, caused temporary accumulation of NO₂⁻-N, a marked change in the nitrification pattern in soil

(6). The fact that individual degradation products of a pesticide can modify the process of nitrification suggests that a more detailed examination of the influence of these types of compounds on other microbial activities in the soil may be necessary.

The effect of pesticides on physiological reactions of microorganisms has been studied much less under anaerobic conditions than in the presence of oxygen. In our laboratory, various pesticides have been investigated to determine their influence on the denitrification process (5), and the present work provides information on the effect of pesticide intermediates on nitrate reduction under anaerobic conditions. Special emphasis was put on the effects of chlordimeform [*N*-(4-chloro-*o*-tolyl)-*N,N'*-dimethylformamidine] and two of its major degradation products, *N*-formyl-4-chloro-*o*-toluidine and 4-chloro-*o*-toluidine (8, 9), alone and in combination. In addition, we attempted to elucidate if changes of the concentration of sand and clay in soil influenced the inhibitory effect of the aniline intermediate 4-chloro-*o*-toluidine.

MATERIALS AND METHODS

We used a Hagerstown silt loam soil with a neutral pH and the following composition: sand, silt, and clay content was 10.5, 66.5, and 23.0%, respectively; organic matter was 1.8%, and the indigenous NO₃⁻-N concentration was 25 μg/g of soil. Before each experiment, the soil was air dried and passed through a 2-mm sieve. Two grams of this soil was placed in an incubation flask, and 1 ml of ethanol containing the desired concentration of the pesticide or its derivatives was added. The control soil was supplied with the same amount of ethanol. After the ethanol was evaporated, 18 g of untreated soil was added to each flask and then all soil was thoroughly mixed on a wrist-action shaker

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for 10 min. Subsequently, 6 ml of distilled water containing 3.5 mg of NO_3^- -N (175 μg of NO_3^- -N/g of soil) was added to each soil sample. To distinguish between biological and physical-chemical activity, control samples were sterilized by autoclaving three times for 45 min at 121°C at 24-h intervals.

Anaerobic conditions were obtained by flushing the flasks with helium. The flasks were incubated for different periods of time, and the gases formed were determined by gas chromatography as previously described (5). Then the soil was extracted by shaking for 30 min with distilled water (40 ml of water per 20 g of soil) on a wrist-action shaker. Separation of soil and aqueous phase was achieved by centrifugation. Nitrate-N and NO_2^- -N in the soil extract were determined with a nitrate electrode (Orion Research, Inc., Cambridge, Mass.) and with the α -naphthylamine-sulfanilic acid procedure (1), respectively. Nitrite interfering with nitrate determination was destroyed by adding 2% sulfamic acid. At least three replicates were evaluated for each treatment, and each experiment was repeated.

Montmorillonite (bentonite) from Clay Spur, Wyo., was obtained from Wards Natural Science Establishment, Rochester, N.Y. To decrease the alkalinity, the clay was treated for 30 min with 1 M CaCl_2 . Then, ethanol was added to a concentration of 70%, and the clay was separated by filtration, thoroughly washed with acetone, and dried at room temperature. The final pH was 7.8 in a 5% clay-water mixture. Sand was purified by exposure to 2 N HCl overnight and, subsequently, intensely washed with distilled water.

Chlordimeform [(*N*-4-chloro-*o*-tolyl)-*N,N'*-dimethylformamide], *N*-formyl-4-chloro-*o*-toluidine, 4-chloro-*o*-toluidine, fluometuron [3-(*m*-trifluoromethylphenyl)-1, 1-dimethylurea], trifluoromethylaniline, metolachlor [2-chloro-2',6'-dimethyl-*N*-(methoxyethyl)acetanilide], 2,6-dimethylaniline, chlorbromuron [3-(3-chloro-4-bromophenyl)-1-methyl-1-methoxyurea], and 3-(4-bromo-3-chlorophenyl)urea were supplied by CIBA-GEIGY Corp.

RESULTS

The effect of chlordimeform on the denitrification process in soil was determined by measuring the disappearance of nitrate, the accumulation of NO_2^- -N, and the formation of N_2O and N_2 . In the untreated control and in the soil supplied with chlordimeform, essentially all the nitrate was converted to nitrogen gas after 36 h of incubation (Fig. 1). Even when 100 ppm (wt/wt) of the insecticide was added to soil, no effect on denitrification was evident when measured at intervals of 16, 24, and 36 h. The amount of nitrate in autoclaved soil did not change during the time of incubation.

N-formyl-4-chloro-*o*-toluidine had no effect on denitrification as compared to the control when it was added to soil at concentrations up to 50 ppm (wt/wt). This observation was made during an incubation period of 36 h with samples taken at 16, 24, and 36 h. However, 100 ppm (wt/wt) inhibited denitrification as indicated by

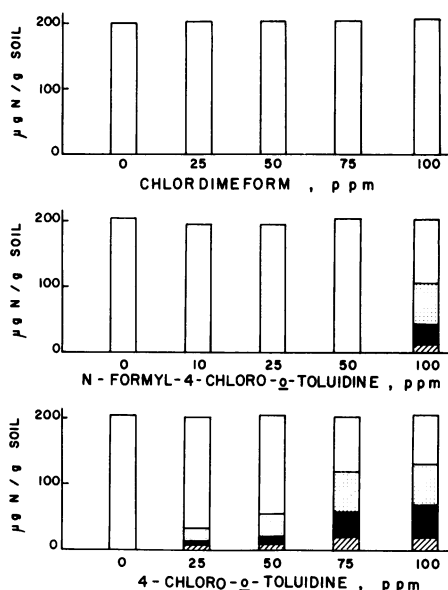


FIG. 1. Effect of various concentrations of chlordimeform and its degradation products *N*-formyl-4-chloro-*o*-toluidine and 4-chloro-*o*-toluidine on denitrification in soil (anaerobic incubation for 36 h at 30°C). Solid parts of bars represent nitrite; hatched parts, nitrate; stippled parts, N_2O ; open parts, N_2 .

the accumulation of nitrite and nitrous oxide and the remaining amount of nitrate as indicated by the measurement after 36 h of incubation (Fig. 1). 4-Chloro-*o*-toluidine was an even stronger inhibitor of denitrification. Increasing concentrations of this aniline in soil caused a corresponding accumulation of nitrite and nitrous oxide. The formation of nitrite and nitrous oxide could also be found in a nontreated soil, but they were transformed to nitrogen gas within a short time. However, in soil to which 100 ppm (wt/wt) of 4-chloro-*o*-toluidine was added, the accumulation of nitrite and nitrous oxide was higher and persisted for a longer time period (Fig. 2).

There is a high probability that after use of chlordimeform the pesticide and its two predominant products, *N*-formyl-4-chloro-*o*-toluidine and 4-chloro-*o*-toluidine, are simultaneously present in soil. When we added two of the chemicals to a soil sample, we found that the combination caused a synergistic inhibition of denitrification (Table 1). For instance, 4-chloro-*o*-toluidine at 50 ppm (wt/wt) caused the formation of 6 μg of nitrite and 34 μg of N_2O per g of soil after 36 h of incubation. However, the addition of 50 ppm (wt/wt) of chlordimeform or 50 ppm (wt/wt) of *N*-formyl-4-chloro-*o*-toluidine in combination with 50 ppm (wt/wt) of 4-chloro-*o*-toluidine resulted in the accumulation of 22 and

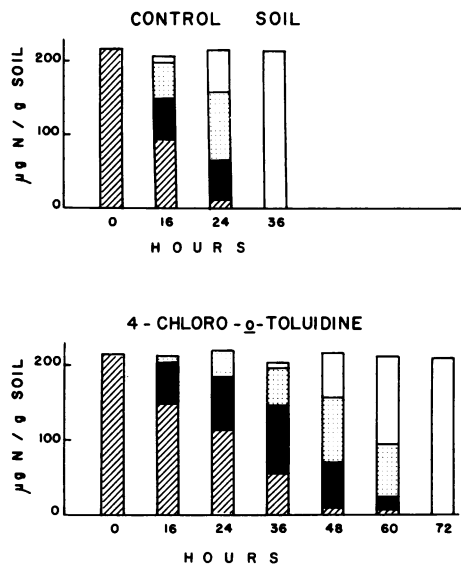


FIG. 2. Denitrification characteristics of soil with and without the addition of 100 ppm (wt/wt) of 4-chloro-o-toluidine. Solid parts of bars represent nitrite; hatched parts, nitrate; stippled parts, N_2O ; open parts, N_2 .

TABLE 1. Effect on denitrification of chlordimeform and two of its degradation products separately and in combination (anaerobic incubation for 36 h at 30°C)

| Treatment (concn [ppm, wt/wt]) | Nitrogen ($\mu\text{g/g}$ of soil) | | | |
|---|-------------------------------------|--------------------|-------------------------|-----------------|
| | As NO_3^- | As NO_2^- | As N_2O | As N_2 |
| Control soil, autoclaved | 223 | 0 | 0 | 0 |
| Control soil, nonautoclaved | <5 | 0 | 0 | 201 |
| Chlordimeform (50) | <5 | 0 | 0 | 197 |
| <i>N</i> -Formyl-4-chloro- <i>o</i> -toluidine (50) | <5 | <1 | 0 | 202 |
| 4-Chloro- <i>o</i> -toluidine (50) | 12 | 6 | 34 | 160 |
| Chlordimeform (25) + 4-chloro- <i>o</i> -toluidine (75) | 25 | 42 | 73 | 69 |
| Chlordimeform (50) + 4-chloro- <i>o</i> -toluidine (50) | 13 | 22 | 48 | 118 |
| Chlordimeform (75) + 4-chloro- <i>o</i> -toluidine (25) | <5 | 9 | 28 | 147 |
| Chlordimeform (50) + <i>N</i> -formyl-4-chloro- <i>o</i> -toluidine (50) | <5 | <1 | 0 | 194 |
| 4-Chloro- <i>o</i> -toluidine (50) + <i>N</i> -formyl-4-chloro- <i>o</i> -toluidine (50) | 12 | 42 | 47 | 93 |
| Chlordimeform (35) + 4-chloro- <i>o</i> -toluidine (35) + <i>N</i> -formyl-4-chloro- <i>o</i> -toluidine (35) | <5 | 14 | 24 | 154 |
| Chlordimeform (50) + 4-chloro- <i>o</i> -toluidine (50) + <i>N</i> -formyl-4-chloro- <i>o</i> -toluidine (50) | 12 | 48 | 69 | 75 |

42 μg of nitrite or 48 and 47 μg of N_2O , respectively.

The observation that the aniline derivative of chlordimeform is a more potent inhibitor of denitrification than the parent insecticide could also be found with other pesticides. The accu-

mulation of nitrite was much higher with the aniline intermediate of fluometuron and metolachlor than with the respective herbicides (Table 2).

Since 4-chloro-*o*-toluidine, a substituted aniline, may easily be bound in the soil, we tried to clarify whether a change of the soil components, which cause different absorption characteristics, influenced the inhibitory effect of the chemical on denitrification. The soil composition was changed by adding different amounts of sand or clay (montmorillonite) to the native soil.

An increase of sand by 40% in the native soils had a slightly inhibitory effect on denitrification (Table 3). Whereas the addition of 10 ppm (wt/wt) of 4-chloro-*o*-toluidine had no effect on denitrification in native soil, it caused a marked inhibition of nitrate disappearance and accumulation of nitrite and nitrous oxide in soil with the increased sand concentration of up to 50%. The addition of 50 ppm (wt/wt) of this compound to soil modified in this way inhibited denitrification to the same degree as 100 ppm (wt/wt) added to the native soil. It has to be emphasized that, with the addition of sand, there was a corresponding decrease of clay and organic matter (see Table 3). Probably the inhibitory effect of sand has to be explained with the concurrent alteration of one of these other components.

Modification of the soil by increasing the concentration of clay through the addition of 5% of montmorillonite had a very slight effect on denitrification (Table 4). However, denitrification was more strongly inhibited in a soil with an increased clay content and treated with 4-chloro-*o*-toluidine at concentrations between 10 and 50 ppm (wt/wt) than soil which was not enriched with clay (Table 4).

DISCUSSION

Individual transformation products of pesticides attract much less attention to their prob-

TABLE 2. Effect of pesticides and their aniline derivatives at a concentration of 100 ppm (wt/wt) on denitrification in soil (anaerobic incubation for 36 h at 30°C)

| Treatment | Nitrogen ($\mu\text{g/g}$ of soil) | | | |
|-------------------------------|-------------------------------------|--------------------|-------------------------|-----------------|
| | As NO_3^- | As NO_2^- | As N_2O | As N_2 |
| Control soil, autoclaved | 210 | 0 | 0 | 0 |
| Control soil, nonautoclaved | <5 | 0 | 0 | 200 |
| Chlordimeform | <5 | <1 | 0 | 207 |
| 4-Chloro- <i>o</i> -toluidine | 21 | 58 | 63 | 72 |
| Fluometuron | 44 | 52 | 41 | 58 |
| Trifluoromethylaniline | 23 | 97 | 32 | 47 |
| Metolachlor | 16 | 30 | 45 | 113 |
| 2,6-Dimethylaniline | 45 | 55 | 34 | 78 |

TABLE 3. Effect of 4-chloro-*o*-toluidine on denitrification in soil modified by increasing sand content (anaerobic incubation for 36 h at 30°C)

| Characteristic of soil ^a | 4-Chloro- <i>o</i> -toluidine added (ppm, wt/wt) | Nitrogen (μg/g of soil) | | | |
|-------------------------------------|--|---------------------------------|---------------------------------|---------------------|-------------------|
| | | As NO ₃ ⁻ | As NO ₂ ⁻ | As N ₂ O | As N ₂ |
| (A) Soil, autoclaved | 0 | 215 | 0 | 0 | 0 |
| (A) Soil | 0 | <5 | 0 | 0 | 202 |
| (B) Soil + 15% sand | 0 | <5 | <1 | <2 | 192 |
| (C) Soil + 40% sand | 0 | <5 | 2 | 35 | 136 |
| (A) Soil | 10 | <5 | <1 | 0 | 195 |
| (B) Soil + 15% sand | 10 | <5 | <1 | 7 | 171 |
| (C) Soil + 40% sand | 10 | 13 | 6 | 56 | 107 |
| (A) Soil | 25 | <5 | 3 | 0 | 177 |
| (B) Soil + 15% sand | 25 | 11 | 28 | 82 | 75 |
| (C) Soil + 40% sand | 25 | 15 | 34 | 81 | 58 |
| (A) Soil | 50 | <5 | 6 | 32 | 167 |
| (B) Soil + 15% sand | 50 | 22 | 36 | 66 | 52 |
| (C) Soil + 40% sand | 50 | 36 | 46 | 53 | 52 |

^a The addition of sand resulted in the following soil composition: (A) soil—10.5% sand, 23% clay, and 66.5% silt; 1.8% organic matter; (B) soil—25% sand, 19.5% clay, and 45.5% silt; 1.5% organic matter; (C) soil—50% sand, 13.9% clay, and 32.1% silt; 1.1% organic matter.

TABLE 4. Effect of 4-chloro-*o*-toluidine on denitrification in soil modified by addition of clay (montmorillonite) (anaerobic incubation for 36 h at 30°C)

| Characteristic of soil ^a | 4-Chloro- <i>o</i> -toluidine added (ppm, wt/wt) | Nitrogen (μg/g of soil) | | | |
|-------------------------------------|--|---------------------------------|---------------------------------|---------------------|-------------------|
| | | As NO ₃ ⁻ | As NO ₂ ⁻ | As N ₂ O | As N ₂ |
| Soil, autoclaved | 0 | 200 | 0 | 0 | 0 |
| Soil | 0 | <5 | 0 | 0 | 207 |
| Soil + 5% montmorillonite | 0 | <5 | 0 | 7 | 214 |
| Soil | 10 | <5 | <1 | 5 | 208 |
| Soil + 5% montmorillonite | 10 | >5 | 4 | 12 | 201 |
| Soil | 25 | >5 | 4 | 0 | 208 |
| Soil + 5% montmorillonite | 25 | >5 | 14 | 57 | 152 |
| Soil | 50 | >5 | 6 | 32 | 169 |
| Soil + 5% montmorillonite | 50 | >5 | 24 | 106 | 95 |

^a The addition of 5% montmorillonite resulted in a soil composition of 28% clay, 9.5% sand, and 62.5% silt; 1.7% organic matter.

able effect on non-target organisms or physiological processes than the parent compound. This oversight has to be emphasized since intermediates are often more resistant in the soil environment, and can cause, as shown in these experiments, a more pronounced effect on a biological reaction than the original pesticide.

Chlordimeform added to soil had no effect on denitrification even at a concentration of 100 ppm (wt/wt), which is 30-fold higher than the recommended rate of application. However, the formyl derivative of toluidine, which is a major decomposition product of chlordimeform under the influence of ultraviolet light as well as in bacterial and fungal metabolism (8, 9), inhibited denitrification at a concentration of 100 ppm (wt/wt), producing an accumulation of 15% of NO₂⁻-N and approximately 30% of N₂O.

Another degradation product, 4-chloro-*o*-toluidine, had an even stronger inhibitory effect under anaerobic conditions. The accumulation of NO₂⁻-N was evident with the addition of only 25 ppm (wt/wt) of this compound to the soil and, at a concentration of 100 ppm (wt/wt), only 25% of the NO₃⁻-N was transformed after 36 h of incubation. The inhibitory effect of 4-chloro-*o*-toluidine is of special interest since the half-life of this compound at 20°C in a liquid medium at pH 7.0 is 4,800 h as compared to 158 h for chlordimeform (CIBA-GEIGY, technical bulletin).

A pesticide and its degradation products are simultaneously present in soil; therefore we investigated the influence of various combinations of chlordimeform and its derivatives on the denitrification process. Whereas 4-chloro-*o*-toluidine added to the soil at a concentration of 50 ppm (wt/wt) caused an accumulation of 3% NO₂⁻-N, its presence in a mixture with chlordimeform or *N*-formyl-4-chloro-*o*-toluidine resulted in an accumulation of 11 and 19% of NO₂⁻-N, respectively.

If anilines are applied to soil, they are mostly bound by both physical and chemical adsorption. We tried to change the adsorption characteristics of soil by adding sand or clay. When 4-chloro-*o*-toluidine was applied at concentrations of 10 to 50 ppm (wt/wt) to soil modified by increasing the sand content to 50% or by an addition of 5% montmorillonite, a marked accumulation of NO₂⁻-N could be seen. The increased inhibitory effect of the aniline in these experiments might be the result of less binding of the chemical to soil. According to Hsu and Bartha (7), soil organic matter is the major constituent in soil responsible for the binding of anilines. Through the addition of sand, for instance, there was a concurrent decrease of organic matter content (Table 3). Therefore, it is perceivable that the decreased organic matter was the reason for a stronger inhibition of 4-chloro-*o*-toluidine. Addition of 5% montmorillonite caused only a minor decrease of the organic matter content. At this time we cannot speculate on the actual reason for the synergistic inhibitory effect between the aniline and the selected clay.

The experiments with soil samples were performed in sealed and oxygen-free incubation vessels. The difficulty of extrapolation from such laboratory experiments to agricultural conditions is obvious, but the temporary accumulation of nitrite and nitrous oxide deserves to be followed in the natural environment for its direct or secondary effects.

Nitrite is usually difficult to detect in routine analysis of field soil which could be explained by its rapid metabolic transformation or its reaction with other compounds. The latter process may be of concern since the reaction with naturally occurring compounds, or in particular with pesticides and other xenobiotics, may result in the formation of nitroso derivatives (3, 12), triazines (11), and other hazardous products. Therefore, the influence of pesticide derivatives on the increased accumulation of nitrite and nitrous oxide, albeit temporary and only shown under artificial conditions, should be explored in nature for its probable environmental impact.

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