Effect of Chemical Structure of Phenylureas and Anilines on the Denitrification Process[1]

by JEAN-MARC BOLLAG and CHERYL L. NASH
Laboratory of Soil Microbiology, Department of Agronomy
The Pennsylvania State University
University Park, Pa. 16802

The widespread use of soil-applied herbicides of the substituted phenylureas, whose effects may persist for several months or years, has given rise to some concern regarding the possible effect of these compounds or their intermediates on the activity of soil organisms. Soil fertility often depends on the very delicate balance which exists between the various types of microorganisms whose activities determine the rapidity and efficiency of the nitrogen, carbon, mineral, and other cycles. Thus, it is clear that the addition to the soil of any potentially toxic molecules constitutes a serious threat to this equilibrium and hence to the future fertility of the soil.

On the other hand, there exists the possibility of considering pesticides not only from a negative or inhibitory aspect, since they may also have a selective and stimulating influence on a part of the microbial soil population. For instance, it can be desirable that the activity of nitrifying bacteria is suppressed if by the conversion of ammonium to nitrate the nitrogen needed for plant growth would be rapidly lost through denitrification or leaching. Contrarily, if there occurs an unwanted accumulation of nitrate it may be beneficial if a certain pesticide provides conditions for extensive activity of denitrifiers. Therefore, tests to establish the possible effect of pesticides or related compounds on soil biota take on great importance.

A considerable amount of information has been compiled on the effects of pesticides on metabolic activities of microbes in soil and on some of the specific processes they perform both in the soil or when grown in pure culture (Fletcher, 1961; Helling et al., 1971). One of the prime concerns in the interference of pesticides in the microbial equilibrium in the soil was related to the nitrogen cycle, and it has been reported that particularly the nitrifying process is susceptible to suppression by pesticides (Prasad et al., 1971). However, very few studies have been made on the pesticidal influence on nitrate reduction or denitrification, although this process also has to be considered as an essential part of the nitrogen cycle.

---

[1]This research was authorized for publication on July 25, 1973 as paper no. 4501 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

Bulletin of Environmental Contamination & Toxicology,
Vol. 12, No. 2 © 1974 by Springer-Verlag New York Inc.

It was our attempt to establish to what extent phenylurea herbicides, and their aniline intermediates as related to their molecular structure influence the process of microbial denitrification.

MATERIALS AND METHODS

A gram-negative, rod-like bacterial soil isolate with denitrifying capabilities, designated Isolate A (Bollag et al., 1970) was used to study the influence of phenylureas and anilines on denitrification. The bacterium was maintained on nutrient agar and then transferred to liquid Giltay's medium and incubated to provide uniform inoculum for the experiments.

To have well-established cultures for the study, the bacterium was introduced into 125-ml Pyrex bottles containing 50 ml of Giltay's medium. This medium consisted of 0.5 g KNO_3 (70 µ NO_3'-N/ml), 1.0 g l-asparagine, 8.5 g Na-citrate, 1.0 g KH_2PO_4, 1.0 g $MgSO_4 \cdot 7H_2O$, 0.2 g $CaCl_2 \cdot 6H_2O$, 0.05 g $FeCl_3 \cdot 6H_2O$ in 1 liter distilled water. The pH was adjusted to 7.2 with NaOH. After inoculation the bottles were closed with one-hole rubber stoppers in which cylindrical rubber septums (Applied Science Laboratories, State College, Pa.) were tightly inserted for gas flushing and gas sampling.

To have anaerobic conditions the bottles were flushed with helium by inserting a syringe needle, connected by tubing to a helium cylinder, into the septum and loosening the rubber stopper temporarily to allow the gas to flow freely. After intermittent flushing for 1 minute the bottles were stoppered tightly and the gas flow reduced until a slight positive pressure remained in the bottles to prevent air contamination. The purity of the helium gas in inoculated bottles was controlled by gas sampling after different time periods and subsequent gas-chromatographic analysis.

The cultures were then incubated for 5 days at 25° C during which time the initial 70 µg NO_3-N/ml medium had been completely utilized. Control bottles having no inoculum were treated in the same way.

After the 5 days of preincubation an additional 70 µg NO_3'-N were added per ml medium in the form of a sterilized KNO_3 solution. The pesticides and anilines were dissolved in 100% ethanol so that 0.5 ml aliquots provided only 1% ethanol and the desired 50, 100, and 200 ppm of pesticide were added after sterilization by membrane filtration (0.22-µm pore size Millipore filter) to 50 ml of the established cultures.

After addition of the pesticides and KNO_3 the bottles were again flushed with helium as previously described, stoppered, and incubated at 30° C for two additional days.

For gas sampling 500 µl gas were withdrawn from each bottle with a gas-tight syringe (Hamilton Corp., Whittier, Cal.) and injected into

a Varian Model 1820 gas chromatograph (Varian Aerograph, Walnut Creek, Cal.) for analysis. Two columns (3 mm O.D., 50° C) were employed: Porapak Q (600 cm, 50-80 mesh) which separated clearly CO_2 and N_2O, and Molecular Sieve 5A (450 cm, 45-60 mesh) which would have indicated the presence of N_2 and O_2. The carrier gas was helium, flowing at 40 ml per min under 42 psi pressure. Dual thermal conductivity cells at 200° C served as detectors. The filament current for Porapak Q was 200mA and for Molecular Sieve 5A 150 mA. The peak areas were integrated automatically (Varian Aerograph Model 477) and the gas quantities calculated from standard gas curves.

Increasing pressure in the bottles resulting from gas production was determined. The amount of gases in excess of normal atmospheric pressure was measured by a manometric device and the total gas volume and specific components were calculated accordingly.

Nitrate disappearance was measured with a nitrate electrode (Orion), and nitrite production was determined colorimetrically by the α-naphthylamine-sulfanilic acid procedure (American Public Health Assoc., 1972).

The following chemicals were obtained from CIBA Agrochemical Co. (Vero Beach, Florida): chlorbromuron, 3-(3-chloro-4-bromophenyl)-1-methoxy-1-methylurea; chloroxuron, 3-[4-(p-chlorophenoxy)-phenyl]-1, 1-dimethylurea; metobromuron, 3-(p-bromophenyl)-1-methoxy-1-methylurea; and technical grade fluometuron, 3-(m-trifluoromethylphenyl)-1, 1-dimethylurea. E. I. Dupont de Nemours Co., Inc. (Wilmington, Del.) provided technical grade diuron, 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea (97.5%); fenuron, 3-phenyl-1, 1-dimethylurea (99.5%); linuron, 3-(3, 4-dichlorophenyl)-1-methoxy-1-methylurea (96.5%); monuron, 3-(p-chloro-phenyl)-1, 1-dimethylurea (98.6%); neburon, 3-(3, 4-dichlorophenyl)-1-butyl-1-methylurea (98.8%); siduron, 3-phenyl-1-(2-methylcyclohexyl) urea (97.3%); and also analytical grade 4-chloroaniline and 3, 4-dichloro-aniline. Technical grade metoxuron, N'-(3-chloro-4-methoxyphenyl)-N N-dimethyl urea (99.8%), was supplied by Sandoz-Wander, Inc. (Homestead, Florida). Two-bromo-aniline, 3-bromo-aniline, and 4-bromo-aniline were obtained from Eastman Organic Chemicals (Rochester, N.Y.). Aldrich Chemical Co., Inc. (Milwaukee, Wis.) supplied 2-chloro-aniline, 3-chloro-aniline, 2,3-dichloro-aniline, 2,4-dichloro-aniline, and 2,5-dichloro-aniline.

RESULTS

Isolate A is a soil bacterium with denitrifying capabilities under anaerobic conditions. It has the characteristic feature of producing the gas nitrous oxide (N_2O) from the reduction of nitrate and, therefore, in this study N_2O was used to measure the degree to which denitrification occurred.

The bacterium was 'precultured' in regular Giltay's medium - as described previously - in order to confine the effect of the added chemicals more on denitrification itself and to avoid the possibility of observing an influence which would be a result of primary effects on other metabolic activities during growth.

It was attempted to clarify to what extent various phenylurea herbicides interfere in the denitrification process. For this purpose different concentrations of the herbicides were added to axenic cultures of the selected bacterium. In the control medium without addition of a herbicide practically all nitrate was volatilized to N_2O within 2 days by an established culture (Table 1). Fenuron, monuron, and metobromuron at a concentration of 50, 100, and 200 ppm had no inhibitory effect on denitrification as indicated by the large amounts of N_2O produced. However, N_2O production was significantly less in flasks containing 50, 100, and 200 ppm of chloroxuron, fluometuron, neburon, siduron, linuron, and chlorbromuron. Diuron and metoxuron suppressed also denitrification, but their effect was less pronounced at 50 ppm. In all samples it was attempted to analyze for nitrite, but this intermediate could not be found as a result of interference by the herbicides.

In each case where no inhibition of denitrification occurred, the phenylurea consisted of only one or no halogen substitution on the aromatic ring, e.g., fenuron, monuron, and metobromuron. However, in every instance where there was a double substitution of halogens on the ring, e.g., chlorbromuron, diuron, linuron, and neburon, there was only partial or no disappearance of NO_3' and a considerably reduced production of N_2O.

It can be seen from the structural formulas of chloroxuron and siduron that these two phenylurea molecules are relatively large due to an additional ring structure, which may explain the resulting inhibitory effect. This also holds true for fluometuron with its large trifluoride substitution on the aromatic ring.

If the denitrification process was reduced or suppressed, it could be observed that the formation of CO_2 increased in a corresponding relationship.

Since anilines are established intermediates in the breakdown of phenylureas, a study was also initiated with this group of compounds. It could be demonstrated that the halogen substituted anilines exert a similar effect in relation to their molecular structure as the investigated herbicides (Table 2). Whereas the anilines with only one chloro or bromo substitution do not affect the denitrifying activity of the investigated bacterium, a double-substitution of a halogen on the aromatic ring brought about a significant inhibition in the formation of N_2O. With the reduction in denitrification it was also possible to observe the formation of some nitrite.

TABLE 1

Effect of various phenylurea herbicides on the denitrifying activity of Isolate A.

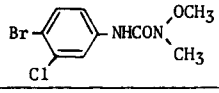
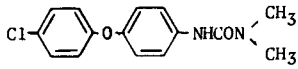
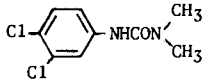
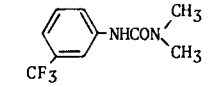
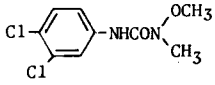
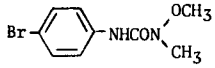
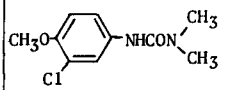
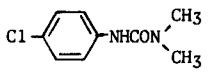
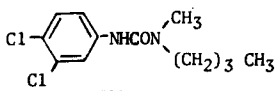
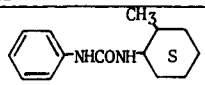
Phenylurea	Structural formula	Concentration in ppm	Nitrogen in µg/ml as NO_3'	Nitrogen in µg/ml as N_2O	CO_2 in µg/ml
Chlorbromuron		50 100 200	52 59 63	14 12 11	20 20 17
Chloroxuron		50 100 200	-- 16 42	-- 45 23	-- 5 16
Diuron		50 100 200	13 55 55	50 16 14	10 16 14
Fenuron		50 100 200	6 4 4	68 67 67	4 5 6
Fluometuron		50 100 200	48 55 57	24 16 15	15 18 18
Linuron		50 100 200	58 60 60	12 12 12	13 14 13
Metobromuron		50 100 200	3 2 2	67 66 68	7 8 7
Metoxuron		50 100 200	4 50 46	65 18 21	4 15 12
Monuron		50 100 200	2 2 2	69 68 66	7 7 7
Neburon		50 100 200	61 59 61	15 12 14	15 14 12
Siduron		50 100 200	51 54 52	17 15 17	17 16 18
Inoculated Control			4	66	8
Non-inoculated Control			70	0	0

TABLE 2

Effect of chlorinated and brominated anilines on the denitrifying activity of Isolate A.

	Nitrogen in µg/ml			CO_2 in
	as NO_3'	as NO_2'	as N_2O	µg/ml
2-Chloro-aniline	3	0	68	6
3-Chloro-aniline	2	0	62	6
4-Chloro-aniline	2	0	64	6
2-Bromo-aniline	3	0	63	5
3-Bromo-aniline	3	0	60	6
4-Bromo-aniline	2	0	57	6
2,3-Dichloro-aniline	39	0.5	29	8
2,4-Dichloro-aniline	42	0.6	35	9
2,5-Dichloro-aniline	28	5.0	36	10
3,4-Dichloro-aniline	21	1.4	50	6
Inoculated Control	5	0	67	5
Non-inoculated Control	70	0	0	0

DISCUSSION

Our results demonstrated that some phenylurea herbicides exert an inhibitive influence on the denitrification process, whereas it was quite obvious that the specific molecular configuration of the compounds was an essential factor in affecting the metabolic process under study. A similar observation was made with chlorinated anilines which are established intermediates of the phenylureas.

It was found that the molecules as characterized by the number of halogen substitution on the aromatic ring, whether it be a phenylurea or an aniline, was correlated to the inhibition of the reduction of nitrate to nitrous oxide. The attachment of two halogens on the aromatic ring of a large single substitution like CF_3 inhibited clearly denitrification while molecules with single chloro or bromo substitutions on the ring had essentially no effect. A relatively large phenylurea molecule like chloroxuron and siduron with one or no halogen substitution showed also some inhibitive effect at the concentrations tested.

An effect of urea herbicides in the nitrogen cycle was only reported in connection with nitrification. It was found that monuron inhibited nitrification temporarily in soil perfusion experiments and pure cultures of Nitrosomonas europaea whereas diuron and neburon did not exert an inhibitory effect (Caseley and Luckwill, 1965). Also Quastel and Scholefield (1953) and Douros (1958) reported that monuron is a "powerful inhibitor of soil nitrification." These observations are in contrast to the reported findings in this paper of the influence of phenylureas during denitrification.

Mitsui et al. (1964) investigated the influence of various dithiocarbamate compounds on the denitrification process in soil samples. They found that mostly Vapam (Sodium methyldithiocarbamate) and Nabam (Disodium ethylenebisdithiocarbamate) inhibited nitrate reduction and gas formation; and they concluded from their investigation that the number of dithiocarbamic 'radicals' in the molecule was not proportional to the inhibitive action of these compounds. However, these investigators did not distinguish to which extent the inhibition was related to a general suppression of the growth pattern or if it was specifically related to denitrification. The use of established microbial cultures appears necessary if one is interested to demonstrate the influence of chemicals on a specific metabolic process.

Although the concentrations of the herbicides used in these experiments are not closely related to the amounts employed in agriculture, the effects of the various compounds on the denitrifying activity indicate clearly the interaction of molecular structure of the chemicals to the metabolic process under study. It is perceivable that an investigation into the connection between the chemical structure of selected compounds and their influence on denitrification may provide a means of regulating by chemical interference the nitrogen cycle in the soil.

REFERENCES

1. AMERICAN PUBLIC HEALTH ASSOCIATION. Standard methods for the examination of water and wastewater. 13th ed. American Public Health Association, Inc., New York. (1972).

2. BOLLAG, J.-M., M. L. ORCUTT, and B. BOLLAG. Soil Sci. Soc. Am. Proc. 34, 875 (1970).

3. CASELEY, J. C., and L. C. LUCKWILL. Rep. Agr. Hort. Res. Sta. Univ. Bristol, pp. 78-86 (1965).

4. DOUROS, J. D. Thesis, The Pennsylvania State University. (1958).

5. FLETCHER, W. W. In "Herbicides and the Soil." E. K. Woodford and G. R. Sagar (eds.), p. 20, Blackwell Scientific Publ., Oxford (1960).

6. HELLING, C. S., P. C. KEARNEY, and M. ALEXANDER. Advan. Agron. 23, 147 (1971).

7. MITSUI, S., I. WATANABE, M. HONMA, and S. HONDA. Soil Sci. Plant Nutrition 10, 15 (1964).

8. PRASAD, R., G. B. RAJALE, and B. A. LAKHDIVE. Advan. Agron. 23, 337 (1971).

9. QUASTEL, J. H., and P. G. SCHOLEFIELD. Appl. Microbiol. 1, 282 (1953).