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Effect of Glyphosate and Nitrapyrin on Selected Bacterial Populations in Continuous-Flow Culture

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The limiting nutrient in the terrestrial environment often controls the overall productivity of that system. Nitrogen is one such nutrient which, under certain conditions, can become limiting to growth. Although the atmosphere provides a nearly endless supply of dinitrogen, most forms of life require nitrogen to be fixed for the synthesis of cellular constituents. Plants can obtain all of their carbon by fixing carbon dioxide, but they are dependent on the availability of inorganic nitrogen and other nutrients present in the soil. Nitrification, the oxidative conversion of ammonium ions to nitrate, produces the principle form of nitrogen assimilated by higher plants. Nitrification, however, is under the control of relatively few genera of bacteria. For these reasons, microbial toxicological procedures based on ecological processes or population testing can be especially useful as measurement endpoints in the assessment of wastes and toxic chemicals.

Traditional laboratory culture of nitrifiers involves the use of static soil cultures or perfusion columns (Lees and Quastel, 1946). Although useful, both techniques suffer from continual changes in substrate concentration and soil chemistry. The continuous-flow method has been used for the culture of heterotrophic soil microorganisms (Hendricks et al., 1987). This technique provides a fixed concentration of nutrients continuously to a soil column and alleviates the limitations mentioned above, and has been used in the culture of nitrifying bacteria (Rhodes and Hendricks, 1990).

This study was designed to use the continuous-flow method to determine the response of nitrifying and heterotrophic bacterial populations to treatments of a known nitrifica-

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tion inhibitor (nitrapyrin) and the widely used post-emergence herbicide, glyphosate. Both nitrapyrin and glyphosate are used extensively in agriculture. Nitrapyrin is commonly used to reduce nitrate **N** loss; while, glyphosate is applied to fields to control unwanted vegetation, but has a weak potential for also affecting nitrification (Carlisle and Trevors, 1986). Based on their known characterstics in routine soil culture, we felt that these two test chemicals would provide an effective and sensitive means to assess the effect of a chemical stressor on an important ecosystem process, nitrification.

Preliminary experiments showed that the continuous-flow method supported high nitrifier activity and was a more sensitive procedure for measuring nitrification than was the soil perfusion and static culture techniques. For this reason, the continuous-flow method was used to examine the effects of chemical treatment on specific genera of ammonium oxidizing bacteria, denitrifying microorganisms, and heterotrophic microbial populations growing within the soil columns.

MATERIALS AND METHODS

Soil bacteria were cultured using the continuous-flow soil columns. Each column was constructed to hold 15 g dry weight Amity soil. This soil is a fine-silty mixed mesic Argiaquic Xeric Argialbolls of the Amity series and is primarily used for agriculture. Since this soil is silty and lacks structure, each column was purged with filtered compressed air to prevent flooding and to provide sufficient aeration to minimize anaerobiosis.

The nitrification medium used in the soil columns throughout this study was based upon that of Page *et al.* (1982) and amended to contain 250 ug NH_4-N mL^{-1}. The medium delivery rate was 10 ml day^{-1}. Effluent samples were collected every two days and analyzed for ammonia (NH_4^+), nitrite ($NO_2^=$), and nitrate (NO_3^-) using a Technicon Autoanalyzer II (Technicon Instrument Company, Tarrytown, ND) and standard EPA techniques previously described (Rhodes and Hendricks, 1990). In separate experiments, the columns were treated with N-Serve 24 {nitrapyrin[1,2]; [2-chloro-6-(trichloromethyl)pyridine]}, Dow Chemical Company, Midland, MI., and Roundup[3] {glyphosate;[N-(phosphonomethyl) glycine]}, (Monsanto Company, St. Louis, MO) on day 8 to insure that the

[1] Mention of trade names or commercial products does not constitute endorsement or recommendation for use.
[2] A gift from Dr. A. T. Talcott, Dow Chemical Company, Midland, MI.
[3] Purchased commercially

columns were actively nitrifying. Nitrapyrin concentrations were 0, 0.042, 0.42, and 4.2 mg active ingredient (AI) g^{-1} dry soil and 0, 0.68, 6.8, and 68 mg AI g^{-1} dry soil for glyphosate. No other carbon source was added, and all experiments were conducted for 16 days. Soil samples were examined to estimate total viable nitrifying, heterotrophic, and denitrifying bacteria (Table 1).

Table 1. Initial microbial populations present in Amity soil

Organisms	Concentration (Log N g^{-1} dry soil)
Heterotrophic Bacteria	
Denitrifiers	8.01 ± 0.15
Soil Extract Agar	8.36 ± 0.15
Starch Casein Agar	8.22 ± 0.15
N-Oxidizing Bacteria	
NH_4 Oxidizers	6.28 ± 0.21
NO_2 Oxidizers	6.46 ± 0.32
Nitrifying Bacteria	
Nitrosolobus	4.99 ± 0.16
Nitrosospira	5.08 ± 0.23
Nitrosomonas	4.05 ± 0.25

Agar plates were inoculated with 0.05 ml of each dilution and culture tubes with 0.5 ml of the dilution series, and nitrifying bacteria were enumerated using a most probable number (MPN) technique (Page et al., 1982; Belser and Schmidt, 1981) as modified by Dr. G. Stotzky (personal communication). Using this modification, organisms were enumerated by incubation in 96 well microtiter plates for six weeks at 25 ± 0.1°C. Positive tubes were then enumerated by comparison to standard five-tube MPN tables. Heterotrophic bacteria were enumerated using plate counts on soil extract agar amended with 1.0% glucose and with starch-casein agar (Page et al., 1982). Denitrifying populations were determined using the MPN method of Focht and Joseph (1973). Tubes containing nutrient broth amended with 1.0 g KNO_3 L^{-1} were inoculated with the decade serial dilutions. Griess-Ilosvay reagents and powdered zinc were used to detect the presence of nitrite and nitrate.

In other studies, fluorescent antibodies (FA) (provided

by Dr. E.L. Schmidt, University of Minnesota) were used for direct microscopic counts of Nitrosomonas (strains SE, europea, and Tara), Nitrosospira (strains AV and Spita), and Nitrosolobus (strains AV, Beardon, and Fargo). The stains within a genus were combined to make antisera specific for those organisms. Soil samples were extracted and prepared for staining following the procedure of Demezas and Bottomley (1986). The microbial extraction efficiency using soils from western Oregon by this technique ranged between 20 and 30%.

Three separate samples were prepared from each of the filtered supernatants. One 10 mL and two 15 mL samples were filtered through three separate Irgalan black stained 0.4 fm pore 25 mm polycarbonate Nucleopore filters supported on a silver membrane, placed on clean microscope slides, counter stained with rhodamine-gelatin (Schmidt, 1974), and fixed by drying at 55°C for 15 min. Slides were viewed with a Zeiss standard microscope (Carl Zeiss, Inc., New York, NY) operating in the epifluorescence mode. For the purpose of enumeration, 25 fields were counted, and the mean and standard error for each specimen was determined.

This research project was designed and managed under an approved quality assurance (QA) project plan as part of the U.S. EPA's Environmental Research Laboratory (Corvallis, Oregon) QA Program. All microbiological assays were prepared in duplicate and analyses were made in triplicate, and an analysis of variance was used to determine significant differences between the treatment means.

RESULTS AND DISCUSSION

The chemical treatment studies in this report focused on the response of nitrifying bacteria native to Amity soil to treatments of nitrapyrin and glyphosate. As a known nitrification inhibitor, nitrapyrin was used as a positive control on inhibition of a nitrifying population by a chemical substance. In this study, inhibition of nitrification was dose-dependent. The values observed were 96.8, 100, and 100% for nitrapyrin and 26.8, 55.9, and 100% for glyphosate (Table 2). Both chemicals did inhibit nitrifying bacteria in this soil, but their numbers were not appreciably changed. The sensitivity to nitrification inhibitors, however, varied greatly among the genera of nitrifying bacteria (Belser and Schmidt, 1981).

The increase in heterotrophic bacteria (Table 2) was probably the result of organic carbon into the system in the form of two treatment chemicals. The numbers of organisms growing on soil extract agar amended with 1.0% glucose, starch-casein agar, and in denitrifier MPN

Table 2. Microbial response to low-level soil treatment with glyphosate and nitrapyrin

Chemical Concentration ($ug\ g^{-1}$ dry soil)	Heterotrophic Bacteria			N-Oxidizing Bacteria		Nitrification Inhibition (%)
	SEA[1]	S-CA[2]	NO_3 Reducers	NH_4	NO_2	
Glyphosate						
0.68	2.34 ± 0.26[3]	0.46 ± 0.07	1.72 ± 0.29	-0.90 ± 0.10	0.00 ± 0.20	26.8 ± 2.3
6.80	3.91 ± 0.22	0.96 ± 0.12	3.45 ± 0.43	-1.80 ± 0.13	-1.01 ± 0.17	55.9 ± 2.8
68.00	1.56 ± 0.36	3.24 ± 0.27	2.59 ± 0.27	0.90 ± 0.18	1.01 ± 0.22	100.0 ± 3.1
Nitrapyrin						
0.042	0.77 ± 0.18	2.99 ± 0.36	0.85 ± 0.12	-1.01 ± 0.17	0.00 ± 0.19	96.8 ± 2.8
0.42	0.77 ± 0.09	3.41 ± 0.41	3.42 ± 0.19	-2.02 ± 0.26	-1.96 ± 0.19	100.0 ± 3.0
4.20	1.54 ± 0.17	8.39 ± 1.09	11.97 ± 1.21	-3.03 ± 0.32	-2.91 ± 0.23	100.0 ± 3.6

[1] Soil extract agar + 1% glucose

[2] Starch-casein agar

[3] Values are expressed as percent change between treated and control cultures on day 16

tubes were significantly greater ($p < 0.05$) than controls in both the nitrapyrin and glyphosate treated soils at 4.2 and 68 mg g^{-1} dry soil, respectively. Significant elution of viable organisms from the column must be considered, but this appeared unlikely as the numbers of heterotrophs increased following treatment. Due to the relative unchanged numbers of ammonium oxidizing bacteria measured by MPN (Table 2), it is believed that although nitrification was inhibited, the organisms did not decrease as one would expect if the compounds were toxic at the concentration applied to the soil. Likewise, the areas under population growth curves estimated by viable MPN and total FA analysis were not substantially different. Generally, MPN estimates are less than FA since only viable cells are enumerated (Tables 2 and 3) and FA analysis stains all cells, whether they are viable or dead. In this study, the inhibition of nitrification cannot be equated with death of the organisms.

Table 3. Effects of glyphosate on selected nitrifying bacteria in soil

Glyphosate [1]	Nitrifying Bacteria		
	Nitrosolobus	Nitrospira	Nitrosomonas
0.68	-2.25 ± 0.21[2]	1.12 ± 0.19	0.00 ± 0.15
6.80	0.00 ± 0.13	0.00 ± 0.16	-1.16 ± 0.11
68.00	1.37 ± 0.11	1.37 ± 0.17	3.93 ± 0.16

[1] Expressed as (ug g^{-1} dry soil)

[2] Values are expressed as percent change between treated and control cultures on day 16.

Nitrosolobus and Nitrosospira were more prevalent than Nitrosomonas among the ammonium oxidizing bacteria present in this soil. Previous studies (Belser and Schmidt, 1978) have found Nitrosolobus to be less numerous than either Nitrosospira or Nitrosomonas in soils examined with these fluorescent antibodies. Nitrosolobus may be ecologically important due to its greater resistance to chemical inhibitors (Belser and Schmidt, 1981). While the MPNs showed a decrease in ammonium oxidizers in treated columns, the FAs, on the other hand, indicated an increase in Nitrosolobus and Nitrosomonas. This discrepancy may be the result of the

strains selected in the FA stains. Since the stains were generated using nitrifying bacteria collected in another geographic area, it could be possible that other strains are unique to the acidic soils of western Oregon. This is due to the diversity of serotypes within genera of nitrifying bacteria (Belser and Schmidt, 1978).

Estimates of doubling times for Nitrosolobus and Nitrosomonas were 8.03 and 15.54 days, respectively. Other data collected in this study indicated that Nitrosospira populations remained essentially unchanged over the incubation period. These values are greater than those reported in other studies (Morrill and Dawson, 1962; Berg and Rosswall, 1987). These data, however, were generated in soils of higher pH and that could, in part, explain the increased generation times observed in this study.

Nitrite oxidizer numbers, as determined by MPN, also decreased in the treated soils. Since nitrapyrin inhibits ammonium oxidation specifically, it is likely that the decrease in nitrite oxidizers was the result of substrate loss. In glyphosate-treated soils the cause is not known and probably not related to nutrient limitation brought about by increased heterotrophic competition for nitrogen sources because of the high levels of ammonium added in the culture medium as described below.

Although a few heterotrophic bacteria capable of nitrification are known to exist, by far the greater number, including those in this study, are obligate autotrophs, are aerobic, use CO_2 as a sole source of carbon, and gain their energy through the oxidation of nitrogen containing compounds. They are unable to use organic substrates. In some of the early studies of autotrophic bacteria, investigators suggested simple carbohydrates were inhibitory to the growth of these organisms. However, these observations have since been proven erroneous. The depression of nitrate content in soil has subsequently been shown to result from the depleation of **N** by organisms requiring organic substrates rather than an effect directly on nitrifing bacteria (Chancy and Kamprath, 1987). It is possible that either the chemical formulations or the soil organic matter affected the nitrification rate attributed to the test chemicals. We do not think that was the case because heterotrophic counts (Table 2) were not sufficiently elevated to substantially reduce **N**, and inorganic **N** was added in excess and regulated by the continuous-flow culture procedure.

The continuous-flow method to culture nitrifying bacteria has proven to be a viable alternative to the traditional methods to culture these bacteria. In this study, the continuous-flow system was used for both nitrification

studies and for studies to determine the impact of chemical compounds on a soil process. The use of the continuous-flow method in this and previous experiments (Hendricks et al., 1987) indicates that it possesses potential for use in other areas of microbial ecology or microbial toxicity assessment.

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