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Factors Affecting the Suppression of *Heterodera glycines* by N-Viro Soil[1]

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Abstract: Previous laboratory research demonstrated that N-Viro Soil (NVS), an alkaline-stabilized municipal biosolid, suppressed plant-parasitic nematodes. This study continued to explore the use of NVS as a nematode management tool specifically addressing factors that could influence its use. N-Viro Soil from different locations, the components of NVS (de-watered biosolids and fly ash admixtures), and sterilized NVS were applied to sand microcosms to determine effects on nematode survival sand solution pH and ammonia concentrations. This study confirmed the previous finding that an important mechanism of *Heterodera glycines* suppression by NVS was the generation of alkaline soil conditions. Only the fly ash admixture that resulted in an increase in pH to 10.0 suppressed *H. glycines* to the same level as NVS. Alkaline-stabilization of biosolids was necessary to achieve nematode suppression. Biosolids applied at rates <3% dry w/w did not suppress *H. glycines* to the same level as equivalent amounts of NVS. Sand solution pH levels after biosolid application, regardless of rate, were approximately 8.5 whereas 1% and 4% w/w NVS amendment resulted in pH levels of 10.3 and 11.6, respectively. NVS from different processing facilities were all effective in suppressing *H. glycines*. The NVS source that produced the highest concentration of ammonia did not reduce *H. glycines* survival to the same level as those sources generating pH levels above 10.1. Microbes associated with NVS appeared not to be responsible for the nematode suppressiveness of the amendment; there was no difference in nematode suppression between autoclaved and nonautoclaved NVS. The role that ammonia plays in the suppression of *H. glycines* by NVS is still unclear.

Key words: ammonia, biosolid, *Heterodera glycines, Meloidogyne incognita,* nematodes, pH.

The N-Viro process mixes de-watered municipal biosolids with alkaline admixtures (i.e., cement kiln dust, fly ash, or quicklime) to yield a pathogen-free, solid material with many beneficial agronomic properties for sale and distribution (Logan and Burnham, 1995). The agricultural value-added benefits of N-Viro Soil (NVS) include improved soil fertility, addition of organic matter, and suppression of plant-parasitic nematodes. More than 100 governmental agencies and private companies use N-Viro technologies to manufacture more than 1 million tons of product annually (www.nviro.com).

In a previous study, NVS amendment suppressed *Heterodera glycines* and *Meloidogyne incognita* equally and second-stage juveniles (J2) of both nematodes (lethal dose required to kill 90% of population [LD_{90}] = 1.4% dry w/w) were more susceptible than the eggs (LD_{90} > 2.6% dry w/w) (Zasada and Tenuta, 2004). In the same study, increasing rates of NVS were correlated positively with pH values in sand solution. Sand solution pH levels, and to a lesser extent the production of ammonia, appeared to be the chemical-mediated factors responsible for killing plant-parasitic nematodes following NVS amendment.

Although the N-Viro process is patented, there can be considerable variability in the NVS produced. Industrial by-products like cement kiln dust and fly ash can be used as alkaline admixtures, alone or in combination, according to local availability and cost (Logan and Harrison, 1994). De-watered municipal biosolids are the solid or semisolid material obtained from treated wastewater. They vary in nutrient content, contaminant content, and pH depending on the source (industrial vs. residential) and treatment methods.

The goal of this study was to investigate factors that may influence the efficacy of NVS as a nematode-suppressive amendment. The specific objectives were to: (i) compare the effects of biosolids and alkaline admixtures used to produce NVS against *H. glycines* (J2), (ii) evaluate NVS from five geographical locations for *H. glycines* suppression, (iii) begin to understand the impact of the microbial component of NVS upon nematode suppression by comparing sterilized and unsterilized NVS, and (iv) assess the role of pH and ammonia upon nematode suppression.

Materials and Methods

Nematode inoculum: Heterodera glycines race 5 from Salisbury, Maryland, and cultured on greenhouse-grown soybean (*Glycine max*) cv. Essex was used for these studies. Four-month-old plants were removed from their pots, soil adhering to the roots was collected, and the root system was agitated in water for several minutes to dislodge cysts. Cysts were extracted from soil and roots (Krusberg et al., 1994) to provide eggs and second-stage juveniles (J2) for subsequent experiments. To obtain J2, cysts were crushed with a glass homogenizer and the resulting solution poured over nested 250- and 25-µm-pore sieves, with eggs retained on the latter. Eggs were allowed to hatch, and J2 were collected and used immediately in laboratory assays.

Assay system: A 2.0 × 2.9-cm polypropylene tube assay assembly described by Zasada and Tenuta (2004) was used in all experiments. Eight cm^3 of sand mixture, with or without amendment, was placed in each assembly. All-purpose sand (Global Stone James River, Buchanan, VA), pH 7.2 in water, was used. The sand was passed through an 850-µm-pore sieve to obtain uni-

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form particle size, washed in distilled water, and dried prior to use. After placing the sand mixture in the tube, 250 to 300 *H. glycines* J2 in 700 µl tap water were added to the top of the sand mixture in the tube. The moisture content of the sand mixture was approximately 60% of water-filled pore space. The tubes were placed in a covered petri dish and incubated on a laboratory bench at approximately 24 °C for 24 hours. The tubes were then placed in 5.0 by 1.5-cm counting dishes to recover surviving J2. Tap water was added to the dishes until water just touched the bottom of the mesh. The recovery units were covered with a glass dish to prevent water loss. Nematodes were extracted from tubes for 48 hours. Thereafter, the tubes were removed, and the number of nematodes that moved through the mesh was quantified directly in the counting dish using a dissecting microscope (Zasada and Tenuta, 2004).

Sand solution analyses: Identical sets of tubes were prepared for each experiment to measure changes in sand solution pH and ammonia concentration after NVS amendment. After the 24-hour incubation period, 1:5 sand:water (w/v) slurries were prepared from all treatments. The mixtures were placed on a reciprocal shaker for 15 minutes and allowed to settle for 15 minutes; the pH of the supernatant was then determined. Ammonium plus concentration of ammonia was measured using a colorimetric method based upon the Berthelot reaction (Rhine et al., 1998). Ammonia concentration in sand solution, expressed as mM, was estimated using the Henderson-Hasselbach equation describing the pH-dependent equilibrium between ammonium and ammonia (Tenuta and Lazarovits, 2002) and moisture content of the sand slurry.

Components of NVS: N-Viro Soil at a 1% and 2% dry weight amendment/dry weight sand (w/w) was compared to the materials used to make NVS at the Toledo N-Viro facility. De-watered biosolid (Toledo, Ohio), Michigan State University (East Lansing, MI) fly ash, and Wyandotte (Wyandotte, MI) fly ash were applied at 53%, 29%, and 18% of the NVS rate, respectively. The final % w/w applied for each material was: 0.2% and 0.4% Wyandotte fly ash, 0.3% and 0.6% Michigan State University fly ash, and 0.5% and 1.1% de-watered biosolid. These rates corresponded to a typical blend ratio used to make NVS at this particular facility. In a separate experiment, de-watered biosolid and NVS were applied at equivalent rates ranging from 1% to 4% w/w. For both experiments, the treatments were mixed into sand at the appropriate specified rates prior to being loaded into the assay container. All materials were applied on a dry-weight basis. Nematode mortality, sand solution pH, and ammonia concentration were measured upon completion of the experiment. For both experiments, treatments were replicated four times and experiments were conducted twice.

Sources of NVS: The nematode suppressiveness of NVS from Daytona Beach, Florida (63% solids, pH 12.2); Leamington, Ontario (68% solids, pH 12.0); Raleigh, North Carolina (65% solids, pH 12.1); Sarnia, Ontario (68% solids, pH 12.5); and Toledo, Ohio (75% solids, pH 12.2) were evaluated. All NVS sources were applied at a 2% w/w rate to sand in microcosms, as this rate was shown to be effective in killing *H. glycines* J2 (Zasada and Tenuta, 2004). Nematode mortality, sand solution pH, and ammonia concentration were measured upon completion of the experiment. Each treatment was replicated four times and experiments were conducted twice.

Sterilized NVS: N-Viro Soil was spread 2.5 cm deep in a container and autoclaved at 122 °C and 0.14 MPa for 30 minutes. Autoclaved and nonautoclaved NVS were applied at a 2% w/w to sand. The same sand solution chemical parameters were evaluated, as described under sand solution analyses, and *H. glycines* J2 mortality was determined. Each treatment was replicated four times and experiments were conducted twice.

Statistical analysis: All nematode mortality data were expressed as a percentage decrease of the number of nematodes surviving in unamended controls. Differences in nematode mortality among treatments were determined by analysis of variance, and means were compared using Tukey's adjustment for multiple comparisons ($P > 0.05$). Nematode mortality data for each replicate were arcsine transformed when necessary to meet the assumption of the statistical models used. The relationship between pH and ammonia concentration measured in sand solution after NVS amendment to nematode mortality was determined using linear least-squares regression models. The fitted models were appropriate, with the Shapiro-Wilk goodness-of-fit for every data set being $w > 0.10$ at $P > 0.01$. Percentage nematode reductions for NVS and biosolid levels were subjected to regression analysis, and differences in the slope between nematode-amendment regression lines were determined using a *t*-test ($P < 0.05$). All errors were presented as ± standard deviation. All data were analyzed using the computer software JMP (SAS Institute, Cary, NC).

Results

Components of NVS: N-Viro Soil and MSU fly ash resulted in similar *H. glycines* J2 reductions, and suppression increased with rate of application ($P < 0.001$) (Table 1). The Wyandotte fly ash and dewatered biosolids did not cause nematode mortality compared to the unamended control. Reduced numbers of *H. glycines* was correlated positively with pH levels measured in sand solution after amendment ($r^2 = 0.74$, $P < 0.01$). There was little relationship between ammonia concentrations and nematode mortality ($r^2 = 0.24$, $P < 0.01$). Only amendment with biosolids produced ammonia; negligible concentrations of ammonia were measured in the other treatments after 24 hours.

TABLE 1. Effects of N-Viro Soil (NVS) and its components on reduction of *Heterodera glycines* juveniles, sand solution pH, and ammonia concentrations after a 24-hour incubation period.[a]

Component	Rate (% dry w/w)[b]	*Heterodera glycines* reduction (%)	Sand solution pH[c]	NH_3 (mM)[d]
NVS	1.0	40 (±9) b[e]	9.4 (±0.2)	0
NVS	2.0	94 (±8) a	10.2 (±0.1)	0
Wyandotte fly ash	0.2	0 (±16) c	7.1 (±0.2)	0
	0.4	0 (±10) c	7.2 (±0.2)	0
Michigan State University fly ash	0.3	25 (±8) b	9.1 (±0.2)	0
	0.6	76 (±14) a	10.0 (±0.4)	0
De-watered biosolids	0.5	3 (±4) c	7.8 (±0.1)	0.1 (±0.1)
	1.1	0 (±9) c	7.4 (±0.2)	0.1 (±0.1)
Unamended sand		0 (±11) c	7.0 (±0.2)	0

[a] Values shown are the average of two experiments with four replications for each treatment ($n = 8 \pm 1$ SD).
[b] Dry weight amendment per dry weight sand. According to the formulation used to make NVS at the Toledo N-Viro facility.
[c] Sand solution pH deteremined on a 1:5 sand:water (w/v) solution.
[d] NH_3 was estimated using the Henderson-Hasselbach equation.
[e] Replicate data were arcsine-transformed prior to PROC MIXED analysis. Values followed by the same letter are not significantly different according to Tukey HSD test ($P < 0.001$).

When NVS and de-watered biosolids were compared at 1% to 4% w/w in sand, all treatments reduced J2 population densities compared with nonamended sand ($P < 0.003$) (Table 2). *Heterodera glycines* mortality was >96% at all NVS rates and increased as the rate of de-watered biosolids increased. The amendments varied in their ability to suppress *H. glycines* with the slopes of the dose-response curves being different ($P < 0.05$). A comparable level of reduction was achieved only when biosolids were applied at ≥3%. The application of biosolids resulted in sand solution pH levels of 8.5 (±0.1), regardless of rate (Table 2). Sand solution pH increased as the rate of NVS increased from 1% to 4%. Less than 0.1 mM of ammonia was produced 24 hours after NVS amendment at any rate. Increase in ammonia concentrations after 1% to 4% biosolid amendment was correlated positively with increased *H. glycines* suppression ($r^2 = 0.88$, $P < 0.01$).

Sources of NVS: There were differences in *H. glycines* suppression among the NVS sources tested ($P < 0.001$) (Table 3). Abundance of *H. glycines* J2 was reduced >95% by NVS from Daytona Beach, Leamington, Raleigh, and Toledo. The Sarnia source of NVS resulted in a 56% (±8) reduction, lower than that observed with the other sources. The percent *H. glycines* reductions observed with the different sources were all different from the unamended control. Raleigh NVS generated the greatest sand solution pH values after 24 hours. There was a linear relationship between sand solution pH measured after NVS source amendment and *H. glycines* mortality ($r^2 = 0.67$, $P < 0.01$). Concentrations of ammonia generated from the NVS sources varied. There was no relationship between ammonia concentration generated after NVS amendment and percent nematode mortality ($r^2 = 0.0$, $P < 0.3$).

Sterilized NVS: Heterodera glycines J2 were suppressed 100% at a 2% amendment rate of autoclaved and non-autoclaved NVS. There was no difference in the pH levels generated after amendment with autoclaved NVS (pH = 11.0 ± 0.3) and nonautoclaved NVS (pH = 11.1 ± 0.2). Very small concentrations of ammonia (<0.1 mM) were produced after the incorporation of autoclaved and nonautoclaved NVS (data not shown).

DISCUSSION

This study confirms findings of Zasada and Tenuta (2004) that the suppression of *H. glycines* by NVS is

TABLE 2. Effects of N-Viro Soil and de-watered biosolids on *Heterodera glycines* juvenile reduction, sand solution pH, and ammonia concentrations after a 24-hour incubation period.[a]

	N-Viro Soil			De-watered biosolids		
% w/w[b]	*Heterodera glycines* reduction (%)	Sand solution pH[c]	NH_3 (mM)[d]	*Heterodera glycines* reduction (%)	Sand solution pH[c]	NH_3 (mM)[d]
0	0	7.3 (±0.3)	0			
1	98 (±4)	10.3 (±0.3)	0	44 (±8)	8.5 (±0.1)	2.3 (±0.6)
2	100	10.9 (±0.5)	0	78 (±4)	8.5 (±0.1)	4.3 (±0.2)
3	96 (±1)	11.2 (±0.4)	0	99 (±1)	8.5 (±0.1)	5.8 (±1.2)
4	99 (±1)	11.6 (±0.4)	0	99 (±1)	8.5 (±0.1)	6.9 (±0.7)

[a] Values shown are the average of two experiments with four replications for each treatment ($n = 8 \pm 1$ SD).
[b] Dry weight amendment per dry weight sand.
[c] Sand solution pH determined from a 1:5 sand:water (w/v) solution.
[d] NH_3 was estimated using the Henderson-Hasselbach equation.

TABLE 3. Effects of N-Viro Soil (NVS) from various geographical locations on *Heterodera glycines* juvenile reduction, sand solution pH, and ammonia concentration after a 24-hour incubation period.[a]

NVS Source	*Heterodera glycines* reduction (%)	Sand solution pH[b]	NH_3 (mM)[c]
Daytona Beach, Florida	98 ± 1 a[d]	11.0 ± 0.3	0.0
Leamington, Ontario	97 ± 1 a	10.2 ± 0.1	0.2 ± 0.1
Raleigh, North Carolina	99 ± 0 a	11.9 ± 0.2	0.1 ± 0.1
Toledo, Ohio	95 ± 5 a	10.2 ± 0.4	0.8 ± 0.5
Sarnia, Ontario	56 ± 8 b	9.9 ± 0.2	6.3 ± 2.9
Non treated control	0 ± 4 c	7.2 ± 0.2	0.0 ± 0.0

[a] NVS was applied to sand at 2% dry weight amendment/dry weight sand. Values shown are the average of two experiments with four replications for each treatment ($n = 8 \pm 1$ SD).
[b] Sand solution pH determined from a 1:5 sand:water (w/v) solution.
[c] NH_3 was estimated using the Henderson-Hasselbach equation.
[d] Replicate data were arcsine-transformed prior to PROC MIXED analysis. Values followed by the same letter are not significantly different according to Tukey HSD test ($P < 0.001$).

primarily a pH-driven phenomenon. Difference in nematode suppression between fly ash sources was due to the pH-raising potential of the material. Sand solution pH was an important factor responsible for the *H. glycines* suppressiveness of the different NVS sources. It appeared there was a threshold soil solution pH that was reached to kill nematodes. Zasada and Tenuta (2004) reported that a pH level generated after 3 hours of 10.2 (resulting from the addition of $Ca(OH)_2$ to sand) resulted in 90% *H. glycines* J2 reduction. In these experiments *H. glycines* was killed when sand solution pH levels exceeded 10.0 after NVS amendment.

It is important to understand the mechanism by which NVS raises soil pH and subsequently suppresses nematodes. Total calcium contents of NVS ranged from 10% to 40% and occurred in different forms, depending on the alkaline reagents used in the stabilization process (Yamakawa, 1999). The following reactions explain the pH-raising potential of NVS:

$$CaO + H_2O \rightarrow Ca(OH)_2 \quad (1)$$

$$Ca(OH)_2 + CO_2 \rightarrow CaCO_3 + H_2O \quad (2)$$

CaO in the alkaline admixture(s) reacts with the water in the dewatered biosolid to form $Ca(OH)_2$ (Eq. 1). Over time, the $Ca(OH)_2$ reacts with CO_2 to form $CaCO_3$ (Eq. 2). $Ca(OH)_2$ has a maximum pH in water of 12.5 and reacts rapidly in the soil. The mean pH value of NVS samples was 11.9, which was controlled by the $Ca(OH)_2$ in the NVS (Yamakawa, 1999). In contrast, the pH of $CaCO_3$ in water is 8.3 and it dissolves slowly, thereby maintaining target pH for periods up to several years (Smith et al., 1998). The presence of the $Ca(OH)_2$ in NVS resulted in a rapid increase in sand solution pH from 7.2 to greater than 10.0, and this pH increase correlated positively with nematode suppression.

This mechanism of nematode suppression was demonstrated when sources of NVS were compared. Sarnia NVS had an initial pH comparable to the other sources, but its inability to suppress nematodes to the same level as the other source may be explained by the form of calcium contained in the material. N-Viro Soil with larger amounts of $Ca(OH)_2$ will maintain pH near 12.0 longer than will biosolids with less $Ca(OH)_2$ but more $CaCO_3$ (Smith et al., 1998). Interestingly, the greatest concentration of ammonia in solution was measured after Sarnia NVS amendment. This was likely the result of the majority of the ammonium in the other NVS sources being rapidly converted to ammonia because of high pH and lost from the system before the 24-hour sampling (Henderson-Hasselbach eq.).

There was no consistent relationship between the amount of ammonia generated after NVS amendment during these experiments and *H. glycines* mortality, refuting our previous observation that the concentration of ammonia measured after NVS amendment was associated with nematode suppression (Zasada and Tenuta, 2004). Ammonia is toxic to nematodes; ammonium nitrate (NH_4NO_3) had both the greatest pH value and the greatest nematicidal activity against *M. javanica* of several ammonium compounds tested (Oka and Pivonia, 2002). *Meloidogyne incognita* J2 were repelled strongly by ammonium salts and ammonium nitrate (LeSaux and Quénéhervé, 2002). The production of ammonia is a dynamic process influenced by temperature, moisture, and soil pH ($pK_a = 9.3$). At pH above 11.0, more than 98% of the ammonium plus ammonia mixture is free ammonia. Fresh NVS has a pH around 12.0, and ammonia was considered to exist as free ammonia (Yamakama, 1999) and may already have dissipated prior to NVS incorporation into soil. The concentration of free ammonia drops rapidly with time due to a decrease in pH and volatilization. Conversely, ammonia appeared to play a role in nematode mortality when dewatered biosolids were applied. Increasing rates of dewatered biosolids, all generating similar pHs, reduced *H. glycines* J2 populations when ammonia was produced. Raw biosolids applied to steam-sterilized soil at a rate of 33% (v/v) reduced tomato galling and *M. incognita* reproduction compared to an untreated control (Castagnone-Sereno and Kermarrec, 1991). It was hypothesized that the lethal effects observed may have resulted from ammonia nitrogen released in the soil after application of sewage sludge. Conversely, plant-parasitic nematodes, as a group, were not affected by heat-treated sewage sludge (Mannion et al., 1994). In this study, comparable concentrations of ammonia were generated when Sarnia NVS or >3% dewatered biosolid were applied, but equivalent nematode mortality was not observed. The role that the production of ammonia after NVS amendment is playing in nematode suppression is still unclear.

At the Toledo NVS facility, MSU fly ash was used as the alkaline admixture and Wyandotte fly ash was used as a bulking agent to facilitate drying of the material.

Fly ash is the residue from coal combustion with an estimated annual production of approximately 60 million tons in the United States (Punshon et al., 2002). Because of the variability in fly ash composition due to the type of coal used, it is difficult to generalize about the potential of fly ash to suppress nematodes. In the present study, MSU fly ash was suppressive to *H. glycines* whereas Wyandotte fly ash was not. In previous studies, fly ash at 0.6 kg/m^2 applied in the row or broadcast decreased the number of galls and egg masses caused by *M. incognita* (Khan and Ghadirpour, 1999) and fly ash extracts reduced *M. javanica* J2 and host penetration and adversely affected post-penetration development (Tarannum et al., 2001). Results of linear regression suggested 40% (v/v) fly ash (pH 9.0) was the level that enhanced yield of infected plants and suppressed *M. incognita* disease and reproduction (Khan et al., 1997).

Although some sources of fly ash suppress plant-parasitic nematodes to the same level as alkaline-stabilized biosolids, the benefits of applying these industrial by-products as a single amendment is an important consideration. Both products have concerns relative to disposal; these concerns are alleviated to a certain extent by combining the materials to produce an agricultural product. The N-Viro process produces a solid, granular material with desirable liming, soil fertility, organic matter, and plant-parasitic nematode suppression benefits. These benefits could not be realized by either by-product alone.

N-Viro Soils manufactured from different source materials and formulations have markedly different properties. For example, Daytona Beach fly ash had a high sulfur content and the ratio used depended upon the solid content of the sewage sludge. The Raleigh facility used 25% quicklime and 25% fly ash for biosolid stabilization. Sarnia NVS was produced from 60% primary (3% to 7% solids, readily settleable biosolids) and 40% secondary (0.5% to 2% solids, activated biosolids) treated biosolids whereas Leamington NVS was produced from 100% primary treated biosolids. Leamington NVS was produced from biosolids containing a large proportion of food-processing waste. Despite the differences in NVS composition from different locations, this research demonstrated that NVS produced from a diversity of by-products has the potential to be an effective component of nematode management systems.

When applying NVS for plant-parasitic nematode management, several factors should be taken into consideration. If alkaline soil conditions are an important component of nematode suppression, then application should be targeted at soils where suppressive pH levels can be generated. The role of ammonia production after the application of NVS and its contribution to nematode suppression is still unclear and needs further investigation. N-Viro Soil containing adequate $Ca(OH)_2$ amounts to facilitate a rapid increase in soil solution pH resulting in nematode suppression should be used. Through time $Ca(OH)_2$ will react with CO_2 to form $CaCO_3$, and the pH of the amendment will decrease.

Various factors that may influence the ability of NVS to suppress *H. glycines* have been addressed. By testing the components of NVS individually it was shown that the alkaline-stabilizing agent of NVS from one location was as lethal to nematodes as NVS and that alkaline stabilization of biosolids was necessary to achieve nematode suppression. The testing of several sources of NVS against *H. glycines* demonstrated that different sources did not vary greatly in their ability to suppress *H. glycines*. $Ca(OH)_2$ levels in NVS may be used to predict the efficacy of the material. Finally, there was no difference in nematode suppression by sterilized and unsterilized NVS, indicating that microbes associated with NVS were not responsible for the nematode-suppressiveness of NVS.

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