

## Mortality of *Pratylenchus penetrans* by Volatile Fatty Acids from Liquid Hog Manure

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**Abstract:** As part of our research program assessing the use of liquid hog manure (LHM) to control root-lesion nematodes, *Pratylenchus penetrans*, a series of acute toxicity tests was conducted to: (i) examine if non-ionized forms of volatile fatty acids (VFA) are responsible for the mortality of *P. penetrans* exposed to LHM under acidic conditions, (ii) determine if *Caenorhabditis elegans* can be a surrogate for *P. penetrans* in screening tests by comparing their sensitivities to VFA, (iii) characterize the nematocidal effect of individual VFA in LHM to *P. penetrans*, and (iv) determine whether individual VFA in LHM interact in their toxicity to *P. penetrans*. LHM was significantly ( $P \leq 0.05$ ) more toxic to *P. penetrans* than a mixture of its main VFA components at concentrations of 5% and 10% (vol. VFA or LHM /vol. in buffer). *Pratylenchus penetrans* was more sensitive to acetic acid than *C. elegans*, whereas the sensitivity of both nematode species to *n*-caproic acid was similar. Individual VFA vary in their lethality to *P. penetrans*. *n*-valeric acid was the most toxic ( $LC_{95} = 6.8$  mM), while isobutyric acid was the least toxic ( $LC_{95} = 45.7$  mM). Individual VFA did not interact in their toxicity to *P. penetrans*, and their effects were considered additive. VFA account for the majority of the lethal effect of LHM to *P. penetrans* under acidic conditions. *Caenorhabditis elegans* cannot be used as a surrogate to *P. penetrans* in toxicity studies using VFA. The efficacy of LHM to control *P. penetrans* can be evaluated by assessing its VFA content prior to application, and this evaluation is facilitated by the fact that the interaction of individual VFA appears to be simply additive.

**Key words:** bioassay, *Caenorhabditis elegans*, LHM, liquid hog manure, LSM, liquid swine manure, management, nematode, potato, *Pratylenchus penetrans*, root-lesion nematode, VFA, volatile fatty acids.

*Pratylenchus penetrans*, (Cobb, 1917) Filipjev and Schuurmans Stekhoven (1941), is considered one of the most economically important plant-parasitic nematodes (Castillo and Vovlas, 2007). This is due to its wide host range, as it has been reported from over 350 hosts (Corbett, 1973) and on every continent except for Antarctica (Castillo and Vovlas, 2007). It is a serious nematode pest of potatoes throughout several potato growing regions in the world (Castillo and Vovlas, 2007), with tuber yield losses estimated to be as much as 20–50% in infested fields (Oostenbrink, 1954). *Pratylenchus penetrans* attacks potato plants causing poor growth and yellowing of the foliage and severe necrosis in roots and tubers (Brodie et al., 1993). The greatest economic damage occurs when the root lesion nematode interacts with the wilt-causing fungus *Verticillium* sp., forming a disease complex known as early dying of potatoes (Martin et al., 1982; MacGuidwin and Rouse, 1990; Rowe and Powelson, 2002). Early dying of potatoes is a significant limiting factor to potato production in various areas in North America (Martin et al., 1982; Rowe et al., 1987).

There is an increasing need to develop environmentally safe and effective alternative strategies to control plant-parasitic nematodes to replace many of the nema-

todes and fumigants that have been abandoned or restricted due to environmental concerns (Rich et al., 2004). Organic amendments hold such potential, and Linford et al. (1938) were the first to report the nematocidal effects of such additions. Since then, application of a range of organic amendments has been found to lead to considerable reduction in plant-parasitic nematode population densities through different mechanisms (Viaene et al., 2006). Application of acidified liquid hog manure (LHM), also known as liquid swine manure (LSM), in microplots and field studies reduced the population of plant-parasitic nematodes including *P. penetrans* below densities that would directly reduce potato yield (Mahran et al., (in preparation)). We were encouraged to determine the nature of suppression of root lesion nematodes by LHM. Short-chain volatile fatty acids (VFA) in LHM have been the focus of several investigations as to how plant-parasitic nematodes and soil-borne plant pathogens have been suppressed. VFA accumulate in LHM after being produced through bacterial fermentation of amino acids which are produced during protein degradation and carbohydrate breakdown either in the gastrointestinal tract of hogs or during their storage under anaerobic conditions (Zhu, 2000).

Non-ionized forms of VFA (acetic, propionic, *n*-butyric, isobutyric, *n*-valeric, isovaleric and *n*-caproic acids) are generated when typically pH-neutral LHM is either acidified or added to acid soil, and it is these forms that were responsible for the death of the microsclerotia of *Verticillium dahliae* (Tenuta et al., 2002; Conn et al., 2005). In addition, VFA in LHM were associated with the suppression of the soybean cyst nematode in greenhouse studies (Xiao et al., 2007). Also, *n*-butyric acid is capable of killing various soil-borne fungi (Browning et al., 2006), both ectoparasitic and endoparasitic plant-parasitic nematodes (Browning

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et al., 2004, 2006) and nematodes from various trophic groups (Browning et al., 2004).

Pre-parasitic juveniles of *P. penetrans* and the standard model nematode, *Caenorhabditis elegans*, share many similar traits. Thus, much of the knowledge that has been gained from *C. elegans* could be transferable to plant-parasitic nematodes (Costa et al., 2007). *Caenorhabditis elegans* has been successively used as a model nematode for plant parasites, especially in elucidating gene functions (McCarter et al., 2003). Advancements that have been achieved in *C. elegans*' neurobiology can be used in studying plant-parasitic nematodes' behavior, especially in neuronal function, which is largely conserved among nematodes (Schafer, 2005). *Caenorhabditis elegans* is a bacterial feeding nematode, 1.0–1.5 mm long, and can be easily maintained in the laboratory in large numbers (Costa et al., 2007). It completes its life cycle in less than three days at 25°C, with an average life-span of two to three weeks, during which it can produce 300 to 350 offspring (Hope, 1999). These traits make it easier to study than *P. penetrans*, which is more difficult to maintain in large numbers in the laboratory because of its obligate plant parasitism and its longer life cycle (Costa et al., 2007). If the sensitivity of *P. penetrans* and *C. elegans* to the VFA in LHM is similar, then *C. elegans* could be used as a simple surrogate to *P. penetrans* in toxicity tests.

As part of our research program of developing LHM to control *P. penetrans*, a series of acute toxicity tests (bioassays) were conducted to: (i) examine if VFA are the compounds responsible for the suppression of *P. penetrans* juveniles exposed to LHM under acidic conditions, (ii) determine if *C. elegans* can be a surrogate for *P. penetrans* in screening tests by comparing their sensitivities to VFA, (iii) determine if the nematicidal effect of individual VFA in LHM varies to the root-lesion nematode and (iv) determine the nature of interaction (additive, synergistic or antagonistic) of individual VFA in their toxicity to *P. penetrans*.

## MATERIALS AND METHODS

**Manure collection and analysis:** In June 2006, LHM was collected from an earthen storage lagoon at a commercial hog farm in south eastern Manitoba, Canada. The manure had a pH of 7.2, 2.7% dry mass, 0.6% total N, 0.01% total P, 0.25% total K, and 2.5 mg NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup>-N/liter fresh manure. The manure was centrifuged (10 min at 3,400xg) to remove particulates. An aliquot of the supernatant was analyzed for its main VFA constituents (C2 to C6 including isomers), while the rest was frozen at -20°C until use in the toxicity tests. Concentrations of individual VFA were determined using chemical suppression ion exclusion chromatography and conductivity detection (Tenuta et al., 2002) using a Dionex ion chromatography system, ICS-1000 (Dionex Corp., Sunnyvale, CA). The chromato-

graph was equipped with an IonPac ICE-AS1 (9 × 250 mm) analytical column and Anion Micromembrane Suppressor (AMMS-ICE II). Diluted LHM (25× in dH<sub>2</sub>O) contained in 1 ml vials with filter caps (Dionex Corp.) were injected (25 µl) using an automated sampler (AS40, Dionex Corp.). For analysis heptafluorobutyric acid 1 mM (Acros Organics Inc.) with 5 % acetonitrile (vol./vol.) was used as the eluent. Commercially available individual VFA (Table 1) were used to calibrate and quantify the samples using an 8 point standard curve.

**Nematode cultures:** *Pratylenchus penetrans* were supplied by Agriculture and Agri-Food Canada (Agassi, BC) and maintained in the laboratory on carrot (*Daucus carota* L.) disk cultures (O'Bannon and Taylor, 1968). After about 8 wk, nematodes were recovered by washing the plates with 10 ml dH<sub>2</sub>O and were collected on a 25-µm pore diam. mesh screen (USA Standard Test Sieve, 500-mesh) for use in the bioassays.

*Caenorhabditis elegans* var. Bristol (wild type strain N2) were provided by the Caenorhabditis Genetics Centre. *Caenorhabditis elegans* nematodes were maintained in 100 × 15 mm sterilized plastic petri plates (Fisher Scientific, Inc.) containing rich nematode growth medium (RNGM) (Tenuta and Ferris, 2004) with a lawn of *Escherichia coli* strain OP50 as a food source. Prior to each bioassay, 200 µl *E. coli* (OP50) L-Broth grown for 24 hr was spread to the surface of a new RNGM plate and incubated at 22°C to grow overnight to form a bacterial lawn. Several hermaphrodites were then transferred to the Petri plates and incubated at 22°C for 5 d. Juvenile nematodes then were harvested by washing the plates with de-ionized water and passing through a 25-µm pore diam. mesh screen.

TABLE 1. Individual volatile fatty acids (VFA) and their commercial product grade, their IUPAC nomenclature and concentration range used in Bioassays 2 and 3.

VFA	IUPAC nomenclature <sup>(1)</sup>	Concentration range (mM)
Acetic acid <sup>(2)</sup>	Ethanoic acid	0, 16, 32, 48, 64, 80, 96 and 112
Propionic acid <sup>(3)</sup> , 99%	Propanoic acid	0, 4, 8, 12, 16, 20, 24, 28 and 32
Isobutyric acid <sup>(3)</sup> , 99%	2-Methylpropanoic acid	0, 8, 16, 24, 32, 40, 48, 56 and 64
<i>n</i> -Butyric acid <sup>(3)</sup> , 99+%	Butyric acid	0, 7, 14, 21, 28, 35, 42, 49 and 56
Isovaleric acid <sup>(3)</sup> , 99%	3-Methylbutanoic acid	0, 8, 16, 24, 32, 40, 48, 56 and 64
<i>n</i> -Valeric acid <sup>(3)</sup> , 99%	Pentanoic acid	0, 2, 4, 6, 8, 10, 12, 14 and 16
<i>n</i> -Caproic acid <sup>(4)</sup> , 99+%	Hexanoic acid	0, 1.5, 3, 4.5, 6, 7.5 and 9

<sup>(1)</sup> International Union of Pure and Applied Chemistry (IUPAC) nomenclature.

<sup>(2)</sup> Sodium Acetate Anhydrous (Fused Crystals/Certified ACS), Fisher Chemical.

<sup>(3)</sup> Source: Sigma-Aldrich.

<sup>(4)</sup> Source: Acros Organics.

*The bioassay:* To conduct the bioassay, 300 nematodes in 0.5 ml dH<sub>2</sub>O were added to 9.5 ml test solution in citric acid-NaOH buffer solution (final concentration of citric acid 23 mM) at pH 4.5 in 15 ml polyethylene test tubes (Fisher Scientific, Inc.). The pH of the test solution was adjusted using 1 M solutions of citric acid and NaOH as needed. A pH of 4.5 was used because at this pH the majority of the VFA will be in their toxic, non-ionized VFA forms (e.g., acetic acid instead of acetate). Preliminary studies showed 300 nematodes yielded a high percentage of recovery (>90%) in the buffered solution at pH 4.5. Nematodes in the test solutions were incubated for 24 hr at 22°C in the dark. To recover the nematodes that survived the test exposure, nematodes were rinsed in tap water three times with centrifugation between each step (5 min at 1,300×g) and decanting to remove any residual test solutions. Afterwards, live nematodes were obtained using a micro-Baermann tube system (Tenuta and Ferris, 2004). Briefly, nematodes were washed into PVC tubes (2.1 cm diam. × 1.4 cm h) opened at one end, with the other end covered with a double layer of Kimwipe laboratory tissue (Kimberly-Clark, Inc.) using a rubber band. The PVC tubes were placed over plastic paper clips to allow some space for the nematodes to move downwards into a gridded counting dish filled with tap water. The dishes with the PVC tubes in them were placed on a tray and covered to reduce water loss and stored at 22°C for 24 hr. The exteriors of tubes and plastic paper clips were rinsed into the gridded counting dishes to dislodge any nematodes adhering on the outside of the extraction tube into the dish. The nematodes in each dish were counted using a dissecting microscope at ×200 magnification. The numbers of nematodes recovered were indexed to the control mean using the following equation: (Numbers of nematodes recovered from each treatment replicate × 100)/(Average number of nematodes recovered in control buffer solution alone). Each treatment had three replicates, and each bioassay was repeated twice. The pH of the test solution was measured using an Accumet pencil-thin epoxy body gel-filled combination electrode with Ag/AgCl reference (Fisher Scientific), and the concentration of non-ionized plus ionized VFA was measured, using the method described previously, before and after the exposure of nematodes to confirm that exposure levels to VFA remained constant over the test period.

*Bioassay 1. Comparing toxicity of LHM and mixture of its VFA to P. penetrans:* To determine if the VFA in the LHM are responsible for the suppression of *P. penetrans*, a mixture of VFA equivalent in concentration to those present in the LHM was prepared using commercially available VFA (Table 1) and compared to LHM in a bioassay. The VFA mixture and the LHM were prepared in a buffered solution of citric acid-NaOH at pH 4.5. The mixture of VFA and the LHM were compared at different concentrations: 0, 5, 10 and 15% (vol. VFA

or LHM/vol. in buffer solution). These concentrations are equivalent to the rate of application of LHM to field soil of 0, 23,400, 46, 800, and 70,200 liters/ha (equal to 0, 2,500, 5,000 and 7,500 US gal/acre) based on a soil moisture content of 30% and a depth of incorporation of LHM in soil to 15 cm.

*Bioassay 2. Relative sensitivity of C. elegans and P. penetrans to VFA:* This bioassay compared the sensitivities of *C. elegans* and *P. penetrans* to increasing concentrations of acetic acid and *n*-caproic acid (Table 1) in a citric acid-NaOH buffer solution at pH 4.5. The final test concentrations used were chosen based on preliminary range-finding toxicity tests (Table 1). The concentration of non-ionized forms in the exposure tests was estimated using the Henderson-Hasselbalch equation (Hasselbalch, 1916), pH of the test solution and VFA (non-ionized plus ionized) concentration. The bioassay was intended to allow the calculation and comparison of LC<sub>50</sub> for both acids to the two nematodes.

*Bioassay 3. Characterization of individual VFA lethality to P. penetrans:* Bioassays to determine the sensitivity of *P. penetrans* to individual VFA present in the LHM were conducted. In these bioassays, *P. penetrans* was exposed to increasing concentrations of acetic, propionic, isobutyric, *n*-butyric, isovaleric, *n*-valeric and *n*-caproic (Table 1) acids in the citric acid-NaOH buffer solution at pH 4.5. The range of concentrations used was chosen based on preliminary toxicity tests using a wide range of concentrations. The concentration of non-ionized forms in the exposure tests was determined as previously described. The concentration of interest was LC<sub>95</sub> (the concentration of individual VFA that killed 95% of the nematodes under study in 24 hr.).

*Bioassay 4. Interaction of individual VFA in the LHM in their toxicity to P. penetrans:* Tests were conducted to assess the role of individual VFA in a mixture scenario to try to understand their potential to interact additively, synergistically or antagonistically in their toxicity to *P. penetrans* when found in LHM. The recovery of live *P. penetrans* in individual test solutions of VFA of concentration equal to an LC<sub>10</sub> was compared to the recovery in a mixture solution of individual VFA with each of them at LC<sub>10</sub> strength.

If the lethality of the mixture of the VFA to the nematodes exceeded the cumulative effect of individual VFA, this was deemed to be an indicator that the interaction between individual VFA was synergistic. However, if the lethality of the mixture to *P. penetrans* was less than that of the cumulative of individual VFA, this was deemed to be an indicator that the interaction between individual VFA was antagonistic. If the toxicity of the mixture of the VFA was equal to that of the cumulative toxicity of individual VFA, this was deemed to be an indicator that the effect of individual VFA was additive.

*Statistical analysis:* Percent nematode survivorship relative to the mean of the control solution (buffer

solution alone) is reported. To calculate the lethal concentrations (LC<sub>10</sub>, LC<sub>50</sub> or LC<sub>95</sub>) and 95% confidence intervals, the data for each replicate from the two experiments were fit to a logistic model using the computer software SAS (Stephenson et al., 2000) using reparameterized models by incorporation of the LC<sub>10</sub>, LC<sub>50</sub> and LC<sub>95</sub> into the equation. The model for estimating LC<sub>10</sub> was:  $y = t / (1 + (0.1/0.9) * (\text{dose}/x)^b)$ ; for LC<sub>50</sub>:  $y = t / (1 + (\text{dose}/x)^b)$ ; and for LC<sub>95</sub>:  $y = t / (1 + (0.95/0.05) * (\text{dose}/x)^b)$ , where  $t$  = survivorship of nematodes in control solution,  $\text{dose}$  = experimental concentration of non-ionized VFA (mM),  $x$  = desired LC value and  $b$  = slope of the curve. The results presented are the average LC<sub>10</sub>, LC<sub>50</sub> or LC<sub>95</sub> of the combined means from the two experiments. Analysis of variance (ANOVA) ( $P \leq 0.05$ ) followed by Tukey's multiple comparisons ( $P \leq 0.05$ ) of the arcsine transformed data was used to compare LHM and a mixture of its VFA in their lethality to *P. penetrans* in Bioassay 1. In Bioassay 2, the sensitivity of *P. penetrans* and *C. elegans* to acetic and *n*-caproic acids was compared using ANOVA ( $P \leq 0.05$ ) followed by Tukey's multiple comparisons ( $P \leq 0.05$ ) of the LC<sub>50</sub> estimated value of individual tested acids. In Bioassay 4, the cumulative effect of individual VFA was compared with a mixture of them at the LC<sub>10</sub> strength using ANOVA ( $P \leq 0.05$ ).

## RESULTS

**LHM analysis:** Five short-chain VFA (from C2-C6) and two isomers were present in the LHM at differing concentrations. Acetic acid was the most dominant VFA in the LHM, having a concentration of 190.3 mM representing more than half the total amount of VFA in the LHM. The LHM used contained nearly equal concentrations of both *n*-butyric and propionic acids, 53.9 and 50.7 mM, respectively, with each one constituting approximately 15% of the total VFA in the LHM. The LHM contained lower concentrations of isobutyric and isovaleric acids, 22.8 and 12.5 mM respectively, representing 3.6 and 6.6% of the total VFA present in the LHM, respectively. *n*-caproic and *n*-valeric acids were present at lower concentrations, 8.7 and 8.5 mM, respectively, each composing about 2.5% of the total VFA in the LHM.

**Bioassay 1. Comparing LHM and a mixture of its VFA in their lethality to *P. penetrans*:** The bioassay results showed that LHM was significantly more lethal to *P. penetrans* than the mixture of its primary VFA (Fig. 1). At the 5% concentration, LHM was significantly ( $P \leq 0.05$ ) more lethal to *P. penetrans* than the mixture of its VFA. LHM killed 60% of the exposed nematodes, and the mixture of its VFA caused 40% mortality (Fig. 1). With the 10% concentration, LHM was still significantly ( $P \leq 0.05$ ) more lethal than the VFA mixture killing 99% of the nematode population, while the VFA mixture was

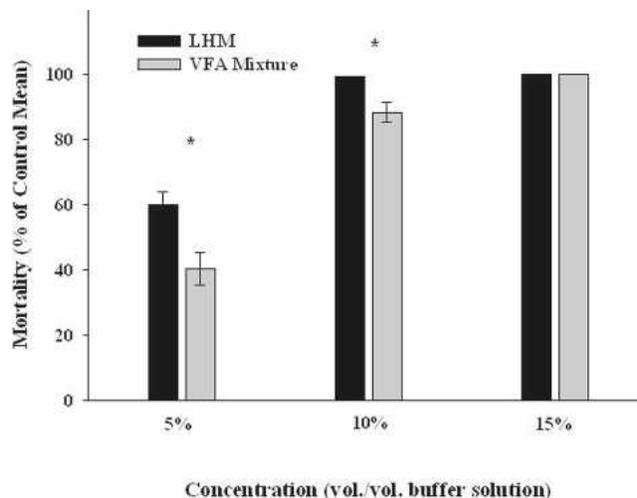


FIG. 1. Results of Bioassay 1 for the comparison of liquid hog manure (LHM) and a mixture of its volatile fatty acids (VFA mixture) in citric acid-NaOH buffered solution (pH = 4.5) in their lethality to *Pratylenchus penetrans*. Shown are the average of two trials with three replicates each ( $n = 6$ )  $\pm$  95% confidence interval. \* indicates statistically significant ( $P \leq 0.05$ ) differences according to Tukey's multiple comparison test using arcsine transformed data.

slightly less lethal, killing 88% of the nematode population relative to the control (Fig. 1). Total mortality of the nematode test population was achieved at 15% (vol/vol buffer) for both the LHM and the VFA mixture (Fig. 1).

**Bioassay 2. Comparison of *C. elegans* and *P. penetrans* sensitivity to VFA:** *Pratylenchus penetrans* and *C. elegans* showed different levels of sensitivity to acetic acid (Fig. 2, Table 2). *Pratylenchus penetrans* was more sensitive ( $P \leq 0.05$ ) to acetic acid than *C. elegans*, with LC<sub>50</sub> values of 8.5 ( $\pm 1.0$ ) and 23.3 ( $\pm 1.4$ ) mM, respectively. The sensitivity of the two nematode species to the larger chain VFA, *n*-caproic acid, was the same ( $P \leq 0.05$ ), with an LC<sub>50</sub> approximate value of 2.3 mM for the two nematodes.

**Bioassay 3. Screening of individual VFA based on their lethality to *P. penetrans*:** Individual VFA were found to vary in their lethality to *P. penetrans* (Table 3, Fig. 3). VFA based on determined LC<sub>95</sub> values to *P. penetrans*, from the most to least lethal, were as follows: *n*-valeric, *n*-caproic, propionic, *n*-butyric, acetic, isovaleric and isobutyric acid (Table 3, Fig. 3).

**Bioassay 4. Interaction of individual VFA in the LHM in their lethality to *P. penetrans*:** The calculated LC<sub>10</sub> concentrations of the individual VFA, acetic, propionic, *n*-butyric, isobutyric, *n*-valeric, isovaleric and *n*-caproic acids, are given in Tables 3 and 4. The sum mortality from individual VFA to *P. penetrans* was not significantly different ( $P \leq 0.05$ ) from the mixture of individual VFA with their concentration set to the calculated LC<sub>10</sub> under our test conditions (Table 4). These results indicate that individual VFA do not appear to be interacting synergistically or antagonistically in their toxicity to *P. penetrans* and that their effect is likely additive.

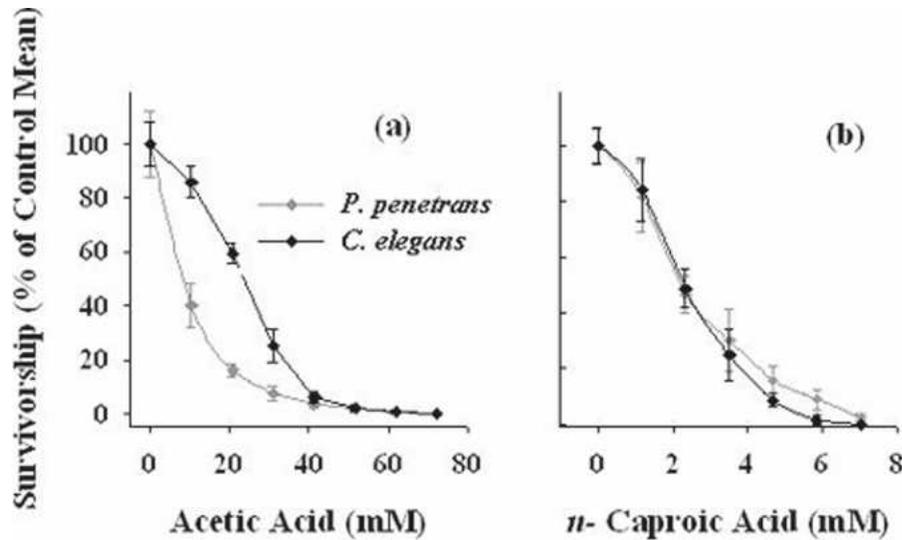


FIG. 2. Results of Bioassay 2 for comparison of the sensitivity of *Pratylenchus penetrans* and *Caenorhabditis elegans* to non-ionized concentration of (a) Acetic and (b) *n*-Caproic acids in citric acid-NaOH buffer solution at pH 4.5. Shown are the average of two trials with three replicates each ( $n = 6$ )  $\pm 95\%$  confidence intervals.

#### DISCUSSION

The primary VFA present in the LHM were acetic, propionic, *n*-butyric, isobutyric *n*-valeric, isovaleric and *n*-caproic acids. The concentrations of individual VFA can vary from one lagoon to another, according to the pigs' diet (Shriver et al., 2003; Lynch et al., 2007), storage conditions (aerobic or anaerobic), and the number and the age of animals in the barn. However, the relative concentration of individual VFA is generally consistent, with acetic and propionic acids dominating, followed by *n*-butyric, isobutyric, *n*-valeric and isovaleric acids at intermediate concentrations and *n*-caproic acid at lower concentrations (Spoelstra, 1980; Tenuta et al., 2002). Our results demonstrated that acetic and propionic acids are the most dominant VFA in the manure (with concentrations of 190.3 and 53.9 mM, respectively) representing more than half the total amount of VFA present in LHM, and these results agreed with those of Spoelstra (1980) and Tenuta et al. (2002). LHM contained lower concentrations of *n*-butyric, isobutyric, *n*-caproic, *n*-valeric and isovaleric acids, 50.7, 22.8, 8.7, 8.5 and 12.5 mM, respectively, which generally agreed with Spoelstra (1980) and Tenuta et al.

TABLE 2. The lethal concentration to 50% of the tested population ( $LC_{50}$ )  $\pm 95\%$  confidence interval of non-ionized forms of acetic and *n*-caproic acids in citric acid-NaOH buffer solution (pH = 4.5) for *Pratylenchus penetrans* and *Caenorhabditis elegans* in Bioassay 2.

		$LC_{50}$ (mM)
Acetic acid	<i>C. elegans</i>	23.3 <sup>a</sup> ( $\pm 1.4$ ) a
	<i>P. penetrans</i>	8.5 ( $\pm 1.0$ ) b
<i>n</i> -Caproic acid	<i>C. elegans</i>	2.3 ( $\pm 0.3$ ) a
	<i>P. penetrans</i>	2.3 ( $\pm 0.2$ ) a

<sup>a</sup>  $LC_{50}$  values of each acid for the two nematodes followed by the same letter are not significantly different from another ( $P \leq 0.05$ ) as determined by Tukey's multiple comparison test.

(2002). The manure used in the present study had 3-fold more *n*-butyric acid concentration than what Tenuta et al. (2002) used (53.9 mM and 16.3 mM, respectively).

Our results demonstrate that non-ionized forms of VFA are the dominant lethal agent in the LHM to the lesion nematode, *P. penetrans*, under acidic conditions. Other factors in LHM seem to contribute to the toxicity observed in *P. penetrans* because the mixture of VFA was slightly less lethal than the LHM itself at 5% and 10%. VFA greater than C6 could be present in LHM, as well as indoles, phenols, volatile amines and sulfur-containing compounds (Zhu, 2000). However, the effect of these compounds on *Pratylenchus* spp. or other pathogens and pests has not been studied. VFA have also been found to be responsible for the control of the microsclerotia of *Verticillium dahliae* (Tenuta et al., 2002) that interact synergistically with *P. penetrans*, causing early dying of potato. However, individual VFA varied in their toxicity to *P. penetrans* and *V. dahliae*. While here *n*-valeric acid was found to be the most lethal to *P. penetrans* ( $LC_{95} = 6.8$  mM), *n*-caproic acid was the most

TABLE 3. Volatile fatty acids (VFA) used in this study arranged according to the lethal concentration of their non-ionized forms to 95% of the tested population ( $LC_{95}$ )  $\pm 95\%$  confidence interval, their  $LC_{50} \pm 95\%$  c.i., their  $LC_{10} \pm 95\%$  c.i., and model parameters b and t.

VFA	$LC_{95}$ (mM)	$LC_{50}$ (mM)	$LC_{10}$ (mM)	b <sup>a</sup>	t <sup>a</sup>
<i>n</i> -Valeric	6.8 ( $\pm 0.6$ )	3.7 ( $\pm 0.2$ )	2.3 ( $\pm 0.2$ )	4.8	100
<i>n</i> -Caproic	8.0 ( $\pm 1.3$ )	2.3 ( $\pm 0.2$ )	0.9 ( $\pm 0.2$ )	2.3	100
Propionic	14.6 ( $\pm 1.2$ )	9.9 ( $\pm 0.3$ )	7.3 ( $\pm 0.7$ )	7.5	100
<i>n</i> -Butyric	25.3 ( $\pm 2.6$ )	8.6 ( $\pm 0.5$ )	3.8 ( $\pm 0.5$ )	2.7	100
Acetic	37.8 ( $\pm 6.8$ )	8.5 ( $\pm 0.9$ )	2.8 ( $\pm 0.7$ )	2.0	100
Isovaleric	39.8 ( $\pm 4.1$ )	16.6 ( $\pm 0.9$ )	8.6 ( $\pm 3.0$ )	3.4	100
Isobutyric	45.7 ( $\pm 4.7$ )	14.6 ( $\pm 0.9$ )	6.2 ( $\pm 0.8$ )	2.6	100

<sup>a</sup> See text for models used and explanation of model parameters.

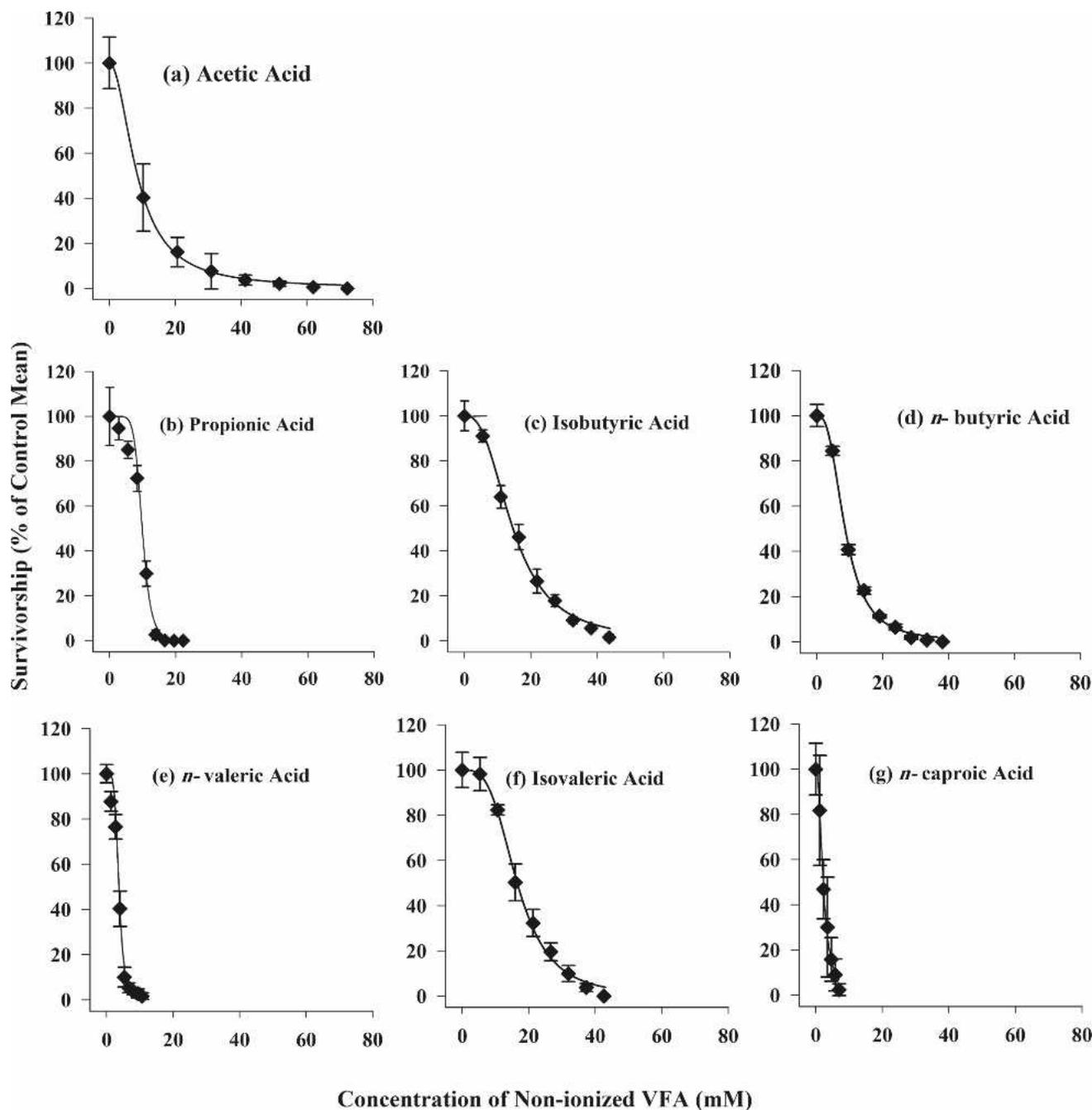


FIG. 3. Results of Bioassay 3 for the mortality of *Pratylenchus penetrans* as a function of the non-ionized concentration of individual volatile fatty acids (VFA) present in the liquid hog manure (LHM), (a) Acetic acid, (b) Propionic acid, (c) Isobutyric acid, (d) *n*-Butyric acid, (e) Isovaleric acid, (f) *n*-Valeric acid and (g) *n*-Caproic acid in citric acid-NaOH buffer solution at pH 4.5. Data shown are the average of two trials with three replicates each ( $n = 6$ )  $\pm 95\%$  confidence intervals. Fitted curves are the logistic models describing mortality of *P. penetrans* as a function of non-ionized concentration of individual VFA.

lethal to *V. dahliae* ( $LC_{95} = 4.1$  mM) (Tenuta et al., 2002). In addition, individual VFA vary in their lethality to the two organisms. For example, acetic acid, which is the dominant VFA in the LHM, was more lethal to *V. dahliae* ( $LC_{95} = 26.2$  mM) (Tenuta et al., 2002) than to *P. penetrans* ( $LC_{95} = 37.8$  mM), while propionic acid, the second-most dominant VFA in the LHM, was more lethal to *P. penetrans* ( $LC_{95} = 14.6$  mM) than to *V. dahliae* ( $LC_{95} = 27$  mM) (Tenuta et al., 2002). Accordingly, the

effectiveness of the LHM in controlling plant pathogens will vary according to the target organism and the VFA profile in the LHM. Information regarding the sensitivity of the target plant pathogen to non-ionized forms of VFA and analysis of the VFA composition of the LHM is essential to predict the effectiveness of the LHM in controlling the target pathogen prior to manure application under field conditions.

*Pratylenchus penetrans* and *C. elegans* have similar sen-

TABLE 4. The lethal concentration to 10% of the tested population (LC<sub>10</sub>) ±95% confidence interval of individual non-ionized forms of volatile fatty acids in citric acid-NaOH buffer solution (pH = 4.5) for *Pratylenchus penetrans* and the percentage nematodes mortality ±95% confidence interval and their cumulative mortality compared to that of the volatile fatty acid (VFA) mixture ±95% c.i. in Bioassay 4.

Treatment	LC <sub>10</sub> (mM) <sup>a</sup>	% Nematode mortality <sup>b</sup>
Acetic	2.8	9.5 ± 5.0
Propionic	7.4	14.1 ± 2.7
<i>n</i> -Butyric	3.8	15.3 ± 2.3
Isobutyric	6.2	8.4 ± 4.4
<i>n</i> -Valeric	2.3	15.6 ± 3.9
Isovaleric	8.6	13.0 ± 3.3
<i>n</i> -Caproic	0.9	7.1 ± 4.0
Cumulative for individual VFA		83.1 <sup>c,d</sup> ± 4.8
VFA mixture		84.7 ± 2.2

<sup>a</sup> LC<sub>10</sub> values determined from previous bioassay.

<sup>b</sup> Percentages are indexed to the control mean.

<sup>c</sup> Cumulative percentage for individual VFA of nematode mortality does not equal sum of individual mortality as shown due to rounding of data.

<sup>d</sup> The cumulative effect for individual VFA is not significantly different ( $P \leq 0.05$ ) from the effect of the VFA mixture according to the ANOVA.

sivities to *n*-caproic acid. However, *C. elegans* showed more tolerance to acetic acid than did *P. penetrans*. The *C. elegans* N2 strain is believed to have been originally isolated from mushroom compost. Like liquid hog manure, composts contain VFA, with acetic acid being dominant and >C4 VFA generally found in very low concentrations (Ozores-Hampton et al., 2001; Bog et al., 2002). Aquino and Santiago-Silva (2006) showed acetic acid persists longer in mushroom compost than propionic, *n*-butyric and *n*-valeric acids (*n*-caproic acid was not detected in the compost). Thus, *C. elegans* may be less sensitive to acetic acid because of its presence in the habitat the culture organism was isolated from originally. The difference in the sensitivity of the two nematodes to VFA indicates that the use of *C. elegans* as a model nematode for the plant-parasitic nematodes in toxicological studies with these compounds is not possible.

In the application of the LHM to a field, VFA are present in the LHM as a complex mixture, and, when applied to the field, *P. penetrans* are exposed to these combinations. Our results show that individual VFA present in the LHM do not appear to have synergistic interactions between them and that their effect is likely additive. This additive effect could be explained by their shared common mode of action, which is the uncoupling of the substrate transport and oxidative phosphorylation from the electron transport system leading to the inhibition of the uptake of essential substrates like amino acids, organic acids and phosphate (Freese et al., 1973). Therefore, it should be possible to predict the overall effectiveness of the LHM to control *P. penetrans*, based on the sum of lethal effects of individual VFA. VFA in LHM was shown to be lethal to *P. penetrans* here and also previously to *V. dahliae* (Tenuta et al., 2002) under acidic conditions. These two pathogens

interact synergistically in their damage to the potato plants, leading to the early dying disease complex (Rowe et al., 1987). That both are killed by VFA indicates great potential of VFA in LHM in controlling the early dying disease complex in acid soils.

In conclusion, our results demonstrate that the VFA in the LHM can account for the bulk of the toxicity observed in *P. penetrans* exposed to LHM under acidic conditions. *n*-valeric acid was the most toxic (LC<sub>95</sub> = 6.8 mM), while isobutyric acid was the least (LC<sub>95</sub> = 45.7 mM). Individual VFA did not appear to interact in their toxicity to *P. penetrans*, and their effects were deemed additive. Our results indicate that *C. elegans* cannot be used as a complete surrogate to *P. penetrans* in toxicity studies using VFA. The efficacy of LHM to control *P. penetrans* can be evaluated by assessing the VFA concentration in the manure prior to application and determination of non-ionized concentration of VFA nematodes are exposed to. This evaluation is facilitated by the fact that the interaction of individual VFA is additive.

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