



Differential effects of butyric acid on nematodes from four trophic groups[☆]

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Abstract

Butyric acid, which is produced through fermentation of organic matter by anaerobic soil bacteria, possesses nematicidal properties. We investigated how the concentration of butyric acid in solution and gas phase affected the survival of 12 nematode species from four trophic groups. Our hypothesis was that survival of free-living and plant parasitic nematodes would differ, since free-living nematodes have shown some adaptation to survival in anaerobic soil environments.

A 2-day incubation in sand amended with 0.88 mg butyric acid g⁻¹ reduced plant parasitic and fungivorous nematodes by 84–100% as compared to untreated controls, whereas a concentration of 8.8 mg butyric acid g⁻¹ was necessary to significantly reduce bacterivorous nematodes (70–98%). Sensitivity of entomogenous nematodes was variable, with *Heterorhabditis* adversely affected by 0.88 mg butyric acid g⁻¹ sand, resulting in a 59% reduction, while *Steinernema* required a concentration of 8.8 mg butyric acid g⁻¹ sand to see a significant decline (85%). Results were similar when nematodes were exposed to the gas phase of butyric acid for 7 days. The vapor from a 0.1 M solution reduced plant-parasitic and fungivorous nematodes by 89–96% while the vapor from a 1 M solution of butyric acid reduced entomogenous nematodes by 94–99%. Bacterivorous nematodes did not survive the 7-day incubation period in appreciable numbers in either controls or treated sand. A 2-day incubation of nematodes in sand acidified with HCl to achieve pH values of 3.4 and 3.0 (similar to sand amended with 0.88 and 8.8 mg butyric acid g⁻¹ sand) had no effect on nematode survival in any of the trophic groups tested. A positive correlation was found between LC₅₀ values for butyric acid and nematode surface area-to-volume ratios for four out of five plant parasitic nematodes ($r = 0.99$; $P = 0.01$) and a negative correlation was found for bacterivorous, entomogenous and fungivorous nematodes combined ($r = -0.77$; $P = 0.07$). The differentiation in chemical tolerances demonstrated here may hold a key to targeting plant parasitic nematodes without affecting free-living forms.

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1. Introduction

Nematodes in soil are functionally diverse. Microbivorous nematodes, those that feed on bacteria and fungi, contribute positively to nutrient cycling and thus plant nutrition. Ferris et al. (1997) estimated N-mineralization rates from grazing nematodes as

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1 $\mu\text{g N g}^{-1}$ soil per day. Chen and Ferris (1999) demonstrated the accumulation of ammonia in soil as a result of nematodes grazing on fungi.

Plant parasitic nematodes cause considerable crop damage with annual losses estimated at US\$ 80 billion worldwide (Handoo, 1998). Breeding for nematode resistance in soybean, potato, tomato and sugar beet is an active area of study (Jung and Wyss, 1999). Synthetic nematicides are used to control nematodes in high-value crops. These substances are environmentally harmful, toxic to the user, and are non-selective, affecting beneficial nematodes as well as the target organisms. Agronomic practices such as crop rotation, bare fallow, cover crops, and soil amendments are used to suppress nematodes. Nitrogen-rich soil amendments result in high levels of ammonia which can plasmolyze nematodes (Rodríguez-Kábana, 1986). Fermentation of organic matter amendments by anaerobic bacteria such as *Clostridium* results in the production of low-molecular weight acids, which have nematicidal properties (Jatala, 1986). These acids include formic, acetic, propionic, and butyric, with butyric acid being the most effective (Johnston, 1959). The large amounts of organic amendments required (tons ha^{-1}) and the constraints on temperature and moisture in soil limit the use of organic amendments to control nematodes (Browning et al., 1999; Noling and Becker, 1994).

Alternate wetting and drying of soils results in a dynamic cycling between aerobic and anaerobic conditions. The ability of soil microbes and fauna to adjust determines their ability to survive such cycles. Nematodes have adaptations enabling them to survive adverse conditions. When oxygen becomes limiting, nematodes may enter a quiescent state in which respiration and metabolism are slowed dramatically. Survival appears to rely on the amount and type of energy reserves stored. *Aphelenchus avenae* was able to recover from 90 days in an O_2 -free environment, compared to less than 80 h for *Caenorhabditis* sp., apparently due to its ability to conserve glycogen (Cooper and Van Gundy, 1970). The free-living nematodes *A. avenae*, *Caenorhabditis* sp. (Cooper and Van Gundy, 1971), and *Panagrellus redivivus* (Butterworth and Barrett, 1985) produce fermentative end products, such as ethanol and acetaldehyde, when held under anaerobic conditions. Barrett (1984) offers a model of the anaerobic pathways utilized by parasitic nematodes. *Steinernema carpocapsae*, an entomogenous

nematode, is also capable of anaerobic metabolism as evidenced by the presence of succinate, acetate, lactate and propionate in nematode extracts incubated under anaerobic conditions (Thompson et al., 1991).

Since free-living nematodes have some adaptation to anaerobic environments, we expect plant parasitic nematodes to be more sensitive to the detrimental effects of butyric acid. If this proves to be the case, butyric acid may have the potential for use as a selective nematicide. We exposed 12 nematode species from four trophic groups to a series of concentrations of butyric acid to determine its toxicity. Nematodes were placed in treated sand or exposed to vapors of butyric acid. The volatility of butyric acid may facilitate its movement through air-filled soil pores, thus increasing its efficacy. In an effort to explain varying sensitivity to butyric acid, we examined the relationship between LC_{50} values for butyric acid and nematode surface area-to-volume ratios, and nematode biomass.

2. Materials and methods

2.1. Nematode sources and culture

The plant parasites *Hoplolaimus galeatus*, *Longidorus sylphus*, *Tylenchorhynchus claytoni*, and *Helicotylenchus robustus* were extracted from golf course greens using Baermann trays. Soil was held at 4 °C prior to use, and nematodes were employed in trials immediately following extraction. A carrot culture of *Pratylenchus penetrans*, originally isolated from potato, was obtained from J. LaMondia, University of Connecticut, Windsor. The nematodes were subsequently maintained on alfalfa root cultured on Gamborg's media (Sigma, St. Louis, MO) with 4% sucrose added. Cultures of fungivorous nematodes were obtained from D. Harshman at Clemson University. *A. avenae* was reared on 1/4 strength potato dextrose agar (PDA) cultures of *Botrytis*, and *Aphelenchoides* sp. was grown on *Cylindrocladium*. The bacterivores *Cephalobus*, *Chiloplacus*, and *Rhabditis* were purchased from Carolina Biological Sciences (Burlington, NC) and reared on sterilized potato plugs in tubes. Nematodes were extracted from cultures with Baermann funnels and used immediately. Commercial preparations of the entomogenous nematodes

Heterorhabditis bacteriophora (Cruiser[®], Ecogen Inc., Langhorne, PA) and *S. carpocapsae* (Millenium[®], Therma TRILOGY Corp., Colombia, MD) were used.

2.2. Exposure of nematodes to butyric acid solutions in sand

Fine sand (0.02–0.2 mm), composed primarily of quartz with some feldspar, was used as the soil medium. These minerals are not likely to sorb butyric acid. Sand was rinsed three times with tap water, and air-dried. Glass vials (20 ml) were half-filled with 10 g dry sand (7 cm³). Butyric acid solutions (1.5 ml) were added to vials to achieve a final concentration of 0.88 µg, 8.8 µg, 88 µg, 0.88 mg, and 8.8 mg each per gram of sand. (Comparable butyric acid concentrations based on soil water content are 5.9 µg, 59 µg, 0.59 mg, 5.9 mg, and 59 mg per milliliter of water).

Acid solutions were stirred into the sand. Distilled water was added to the control vials. Approximately 150 nematodes (with some exceptions) in a 100-µl suspension were added to the sand surface in each vial. The bacterivores, *Chiloplacus*, *Rhabditis*, and *Cephalobus*, did not survive well over time in the sand medium, so concentrations were increased to 400 nematodes per vial. Since *Longidorus* were available only in very limited quantities, 20 nematodes were handpicked and added to 0.5 ml of water in vials prior to filling with treated sand. Each concentration was replicated five times. Vials were capped and held at room temperature (18 °C) for 2, 4, or 7 days. At the end of the incubation period, the vial contents were transferred to Baermann funnels for nematode extraction. Nematodes were collected 16 h later and counted.

2.3. Exposure of nematodes to butyric acid in the gas phase

Nematodes, held in sand in PVC cylinders, were exposed to vapors of butyric acid. Cylinders were constructed from PVC tubing (2.1 cm i.d.) cut into 2.5 cm lengths. Nitrile fabric (20-µm-mesh; Sefar America, Inc., Depew, NY) was attached to one end with a rubber band. The cylinders received 10 g moistened sand (13%, v/w). A nematode suspension (100 µl) was pipetted onto the sand surface, delivering

approximately 150 nematodes. As in the vial experiment, rates of 400 bacterivores and 20 *Longidorus* were used.

Polypropylene storage containers (41, 28 cm × 18 cm × 8 cm) were used to contain the butyric acid vapors. Two glass petri dishes, placed in each container, received 10 ml of butyric acid solution (0, 0.1 mM, 1 mM, 10 mM, 0.1 M, and 1 M). A plexiglass sheet in which holes (2 cm diameter) had been cut was suspended 2 cm above the butyric acid solutions. Five cylinders containing nematodes in sand were positioned over the holes. The plastic containers were sealed and left undisturbed for 7 days at room temperature (18 °C). Nematodes were then extracted with Baermann funnels and counted. Absorption of butyric acid by the materials used in this study was thought to be minimal, and was consistent for all nematode species tested.

2.4. Effect of inorganic acids on nematodes

To evaluate the effect of acidification on nematodes in the absence of butyric acid, hydrochloric acid (HCl) was employed to simulate the pH values in sand following the addition of butyric acid. Sand amended with 0.88 and 8.8 mg butyric acid g⁻¹ had a pH of 3.5 and 3.0, respectively. To mimic these pH values, solutions of 0.001, 0.005, and 0.01N HCl were added to sand in vials, resulting in pH values of 3.9, 3.4, and 3.0, respectively. Nematodes were added as described previously. Treatments were replicated five times. Following a 2-day exposure, nematodes were extracted and counted.

2.5. Relationship between LC₅₀ for butyric acid and nematode surface area-to-volume ratios or nematode biomass

Female nematodes were hand picked and fixed in a solution of 10 ml formalin, 10 ml glacial acetic acid and 80 ml distilled water heated to 100 °C (Hooper, 1970). For *Heterorhabditis* and *Steinernema*, the free-living infective juvenile stage was employed. Ten nematodes were measured per species. Nematode width (*W*), volume (*V*), and biomass were computed from nematode length (*L*) and projected area (measured using an Axiovision image analysis system; Carl Zeiss, Thornwood, NY). Nematode surface area

was calculated with the formula, $L(2\pi*W/2)$, and nematode biovolume was computed assuming a circular cross-section using the formula $V = \pi(W/2)^2L$. Biomass was computed using the method of Andr assy (1956).

LC₅₀ values (concentrations required to kill 50% of the nematode population at the 2-day evaluation) were determined using the inhibition concentration (IC) approach, a method which uses linear interpolation to calculate a point estimate (inhibition concentration) of a toxicant that causes a percent reduction (Norberg-King, 1993).

2.6. Statistics

Data for exposure to butyric acid in sand were subjected to a two-way factorial analysis, and data for exposure of nematodes to the vapor phase of butyric acid and inorganic acid in sand were subjected to one-way ANOVA. Tukey's multiple comparison test identified significant differences between means.

Pearson correlation analysis was applied to nematode LC₅₀ values for butyric acid and nematode surface area-to-volume ratios as well as to nematode biomass.

3. Results

3.1. Exposure of nematodes to butyric acid solution in sand

Concentration of butyric acid was a significant factor for all nematode species tested ($P < 0.01$). The length of exposure was a significant factor ($P < 0.01$) for all except the plant parasite *Hoplolaimus* and the entomopathogen *Steinernema*. However, extending the period of exposure to butyric acid from 2 to 4 or 7 days had no significant impact on the efficacy of butyric acid at any concentration. Therefore, the effect of butyric acid concentration on nematode survival within the 2-day exposure period only is reported.

A 2-day incubation in sand treated with 0.88 mg butyric acid g⁻¹ was sufficient to reduce plant parasitic nematode densities by 94–100%, and fungivorous nematodes by 70–95% relative to untreated controls (Fig. 1). The plant parasitic species, *Hoplolaimus* and *Tylenchorhynchus*, were also affected adversely by exposure to 88 µg butyric acid per gram of sand (73 and 59% reductions, respectively). Bacterivorous nematodes were less sensitive to butyric acid, with only the highest concentration (8.8 mg g⁻¹)

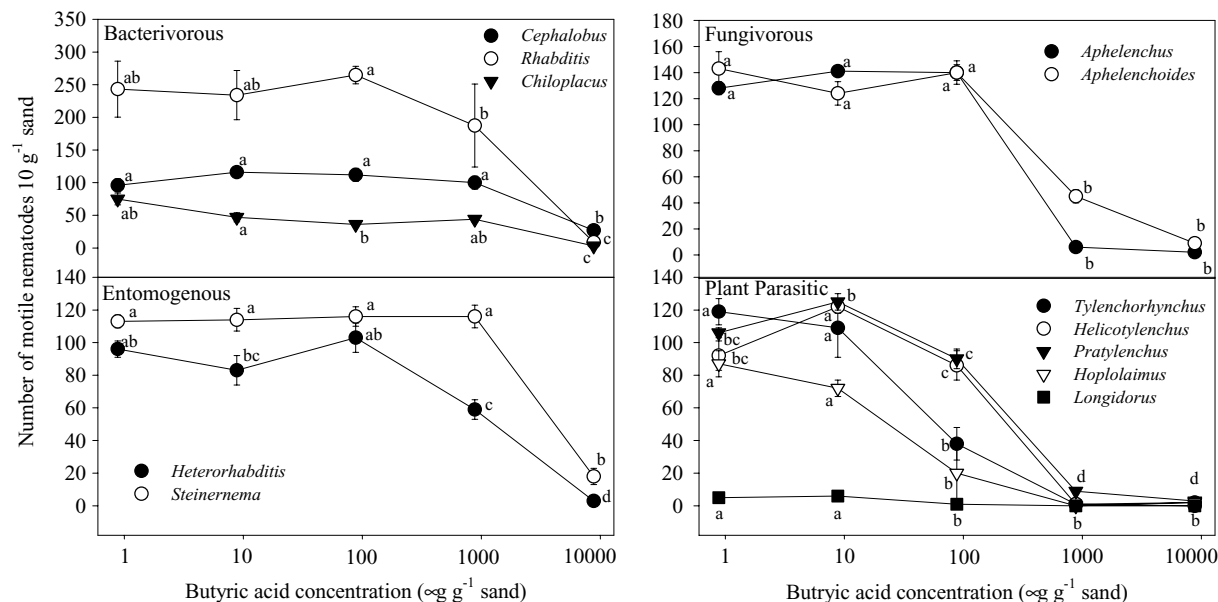


Fig. 1. Number of motile nematodes \pm S.E.M. following 2 days in sand amended with butyric acid ($n = 5$). Means denoted with the same letter are not significantly different ($P < 0.05$).

resulting in a significant decline in nematodes (70–98%). Sensitivity of entomogenous nematodes was variable. *Heterorhabditis* was reduced 52% by exposure to 0.88 mg butyric acid while *Steinernema* required a concentration of 8.8 mg butyric acid to see a significant reduction (85%).

3.2. Exposure of nematodes to butyric acid in the gas phase

Exposure of nematodes to vapors of butyric acid for a period of 7 days yielded the same results as when nematodes were incubated in sand treated with butyric acid (Fig. 2). The two solutions with the

highest concentrations of butyric acid (0.1 and 1 M) resulted in a significant reduction in plant parasitic and fungivorous nematodes (89–100%) as compared to the untreated controls. Entomogenous nematodes were adversely affected only by the highest rate, with a reduction of 94–99%. Bacterivorous nematodes did not survive the 7-day exposure period in appreciable numbers in either controls or treatments.

3.3. Effects of inorganic acid on nematodes

Acidification of sand with HCl (pH 3.0–3.9) did not reduce population densities in any of the nematode

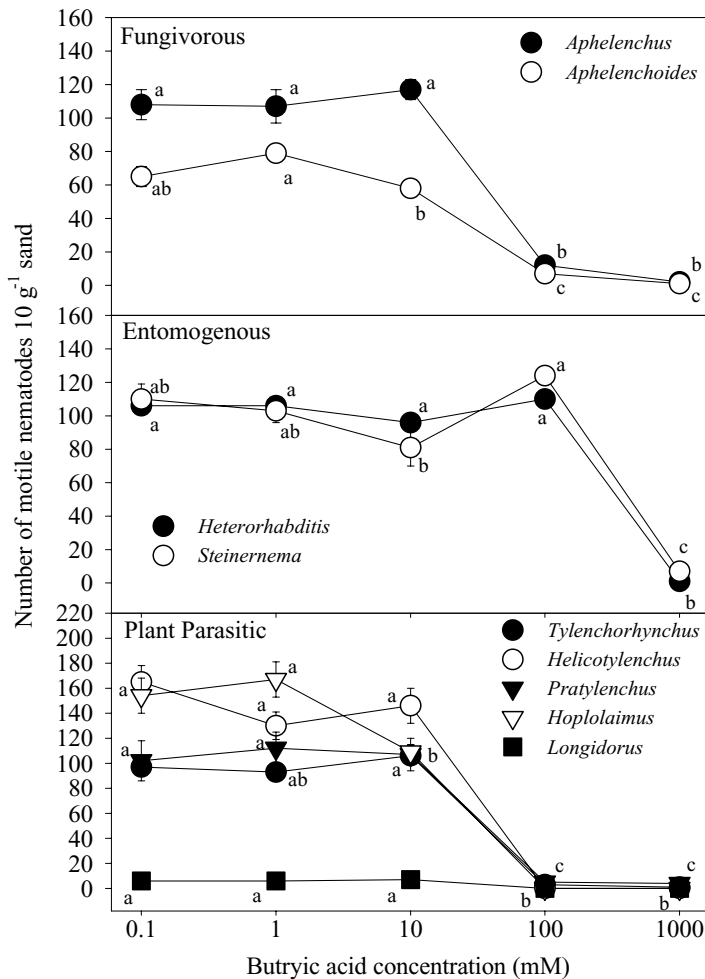


Fig. 2. Number of motile nematodes ± S.E.M. following 7-day exposure to vapors of butyric acid (n = 5). Means denoted with the same letter are not significantly different (P < 0.05).

species following 2-day exposure relative to untreated sand (pH 4.8).

3.4. Relationship between LC_{50} for butyric acid and nematode surface area-to-volume ratio

LC_{50} values ($\mu\text{g g}^{-1}$ sand) for butyric acid ranged from 3 to 30 for plant parasitic nematodes; 50 and 60 for the fungivores; 90 and 537 for the entomogenous nematodes; 380 and 600 for the bacterivores. A positive correlation between nematode surface area-to-volume ratios and LC_{50} for butyric acid was found for four out of five plant parasitic nematodes ($r = 0.99$; $P = 0.01$) (Fig. 3). When *Helicotylenchus* was included in the analysis, the correlation coefficient was 0.66 ($P = 0.22$). A negative correlation was found between nematode surface area-to-volume ratios and LC_{50} for butyric acid for bacterivorous, entomogenous, and fungivorous nematodes combined ($r = -0.77$; $P = 0.07$). There was no apparent relationship between LC_{50} for butyric acid and nematode biomass.

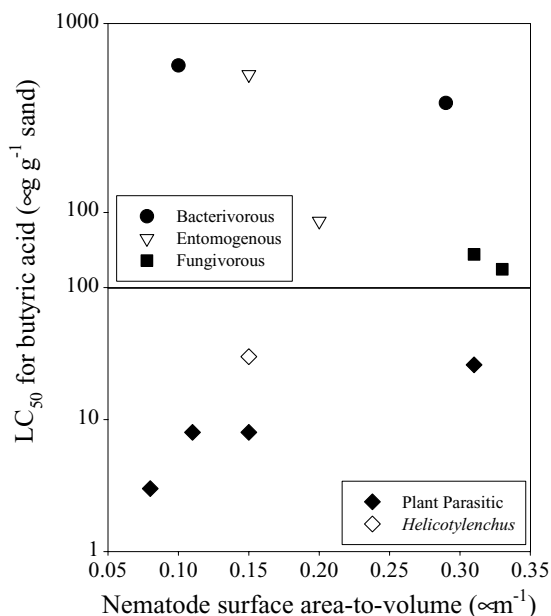


Fig. 3. Correlation between nematode surface area-to-volume ratios and their corresponding LC_{50} values for butyric acid for bacterivorous, entomogenous and fungivorous nematodes combined ($r = -0.77$; $P = 0.07$), and plant parasitic nematodes, excluding *Helicotylenchus* ($r = 0.99$; $P = 0.01$).

4. Discussion

A rate of $0.88 \text{ mg butyric acid g}^{-1}$ sand reduced plant parasitic nematodes by 94–100% following a 2-day exposure. Comparable field rates, assuming a treatment depth of 15 cm, would be approximately 1916 kg ha^{-1} . Preliminary field trials, however, have demonstrated a rate of $671 \text{ kg butyric acid ha}^{-1}$ to be as effective as a synthetic nematicide in suppressing root knot nematodes (*Meloidogyne hapla*) on tomatoes and lesion nematodes (*P. penetrans*) on strawberries (Mitkowski and Jordan, 2003). McElderry (1998) also reported increased efficacy when *Tylenchorhynchus* spp. were held in soil amended with butyric acid.

Functional groups of nematodes exhibited differential tolerance to butyric acid in solution and gas phase. Bacterivorous nematodes were less sensitive to butyric acid than were fungivorous nematodes. Fungivores, in turn, were less sensitive than plant parasitic nematodes. These results mirror the findings of Dijan et al. (1994) who reported TC_{50} (toxic concentration) values for a variety of nematodes following a 24 h incubation in a solution of pentanoic acid.

Differences in response of trophic groups to butyric acid doses may be explained by several factors relating to adaptations to soil environmental conditions and morphological differences between species. Soils are spatially heterogeneous habitats whose oxygen state changes dynamically. Depending on texture and structure of soils, variations in soil moisture content over time may affect nematodes adversely. For example, soil aggregates are considered habitat for micro- and meso-fauna. The centers of aggregates may become anaerobic even when the bulk soil is aerobic, thus subjecting nematodes to alternating aerobic and anaerobic conditions.

A. avenae, a fungivorous nematode, and the bacterivores, *Caenorhabditis* sp., and *P. redivivus*, produce fermentation end products when placed under anaerobic conditions (Cooper and Van Gundy, 1971; Butterworth and Barrett, 1985) suggesting that they are capable of anaerobic metabolism. These organisms are able to metabolize fermentation end products when aerobic conditions are re-established. Similarly, the entomogenous nematode *S. carposcaphsae*, whose free-living juvenile form migrates through the soil in search of a host, is capable of anaerobic metabolism (Thompson et al., 1991). Physiological

adaptations to anaerobic conditions, however, may not protect free-living nematodes from the effect of butyric acid, since *A. avenae*, a fungal feeder capable of switching to anaerobic metabolism, was more sensitive to butyric acid than the bacterial feeders.

Plant-parasitic and fungal feeding nematodes may avoid the deleterious effects of anaerobic conditions found in soil because they feed on plant roots and fungal hyphae, which can be conduits for gases such as oxygen. However, the rhizosphere itself may have reduced O₂ partial pressures because the high abundance of microorganisms active in the root zone may deplete it of oxygen (Gisi et al., 1997).

Could the differential effects of butyric acid be explained by morphological differences between nematodes? Atkinson (1980) observed that oxygen consumption rates in nematodes are dependent on body weight. Our results showed that the LC₅₀ for butyric acid was not weight-dependent. If the amount of butyric acid that is taken up by nematodes is dependent on the surface area of the organisms, then a high surface area-to-volume ratio would increase the surface area through which protonated forms of butyric acid (Banage and Visser, 1965) can diffuse relative to the body mass that would have to tolerate and metabolize it. We hypothesized that the tolerance of nematodes decreases as the surface area-to-volume ratio increases. This trend was apparent in all trophic groups except for the plant parasites (Fig. 3). We are not aware of a physiological explanation for the outlier status of *Helicotylenchus*, but the other plant parasitic nematode species showed a strong positive correlation between LC₅₀ for butyric acid and surface area-to-volume ratios.

Butyric acid is a naturally occurring organic acid produced by fermentative bacteria in soil. The microbial community quickly metabolizes butyric acid under aerobic conditions, so any residual effect from its application should be short-lived. Its volatility will allow for rapid diffusion through the air-filled soil pores. Due to the relative tolerance of bacterivorous nematodes to butyric acid as compared to plant parasitic nematodes, use of this material as a nematicide may result in less disruption to the free-living nematode community than results from the applications of synthetic soil sterilants.

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