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# Persistence of Four *Heterorhabditis* spp. Isolates in Soil: Role of Lipid Reserves[1]

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*Abstract:* Infective juveniles of four *Heterorhabditis* isolates (*H. bacteriophora* HI, *H. megidis* UK211 and HF85, and *H. downesi* M245) were stored in moist (pF 1.7) and dry (pF 3.3) loam soil at 20 °C for up to 141 days. Survival, assessed by the number of nematodes extracted by centrifugal flotation, declined over time, reaching fewer than 18% alive by day 141 for all but one treatment (*H. bacteriophora* HI in dry soil). The infectivity of nematodes in soil for *Tenebrio molitor* also declined over time, roughly in accordance with the decline in numbers of nematodes. Energy reserves of extracted nematodes were assessed by image analysis densitometry. There were differences among isolates both in survival and in the depletion of reserves, and there was a significant correlation between these two parameters, suggesting that the extent to which energy reserves are depleted affects survival or that a common factor influences both. However, significant nematode mortality occurred while levels of reserves remained high, and the maximum reduction in utilizable body content for any treatment was 51%, well above starvation level. Therefore, the decline in numbers of living nematodes and the reduced nematode infectivity in soil cannot directly result from starvation of the nematodes. Survival and infectivity declined more rapidly in moist than in dry soil; one isolate, *H. downesi* M245, was less affected by soil moisture content than the other three isolates.

*Key words:* energy reserve, entomopathogenic nematode, *Heterorhabditis bacteriophora, Heterorhabditis downesi, Heterorhabditis megidis,* infectivity, persistence, soil baiting, starvation, survival.

The infective juveniles (IJ) of parasitic nematodes are capable of surviving for weeks or even months without any exogenous source of energy (Croll and Matthews, 1977). They depend exclusively on their accumulated energy reserves. Where conditions are otherwise favorable, the duration of survival is determined by the amount and rate of utilization of the reserves. Lipids, especially neutral lipids, are the main energy reserve (Barrett and Wright, 1998) and may account for a considerable proportion of the body contents. In the IJ of the entomopathogenic nematodes *Steinernema* and *Heterorhabditis,* lipids constitute 32% to 43% of total body weight (Fitters et al., 1999; Selvan et al., 1993). There is evidence that starvation is the main cause of mortality for entomopathogenic nematode IJ in water (Fitters, 1999; Qiu and Bedding, 2000). Motility and infectivity have been correlated with depletion of reserves in entomopathogenic nematodes stored in water or moist sponge (Lewis et al., 1995; Patel et al., 1997; Vänninen, 1990).

Depletion of energy reserves in soil has not been studied for any species of entomopathogenic nematode, though some researchers have assumed that abiotic factors such as temperature, soil moisture, and texture influence survival and infectivity partly by affecting the rate of utilization of reserves, especially lipids (e.g., Kung et al., 1990, 1991; Molyneux, 1985). Hass et al. (2001) found that the persistence of 10 strains of *Heterorhabditis* in soil was significantly correlated with their persistence in water, suggesting that the rate of utilization of energy reserves may be an important determinant of strain-specific soil persistence.

The aim of the present study was to investigate to what extent a decline in survival and infectivity of *Heterorhabditis* IJ in soil is correlated with a decline in stored energy reserves, and whether differences among nematode strains in rates of energy depletion could account for observed differences in persistence in soil.

## Materials and Methods

*Nematodes:* Four isolates were used, representing three species of *Heterorhabditis: H. bacteriophora; H. megidis* North West European type (Smits et al., 1991); and the newly described species (Stock et al., 2002) *H. downesi,* formerly known as Irish type *Heterorhabditis* (Joyce et al., 1994; Smits et al., 1991). *Heterorhabditis bacteriophora* HI originated in Italy; *H. megidis* UK211 and HF85 originated in England and the Netherlands, respectively; and *H. downesi* M245 originated in Ireland. Nematodes were cultured in late instar larvae of *Tenebrio molitor.* Harvested IJ were washed three times by sedimentation in tap water and stored in tap water at 9 °C for 12 days before application to soil.

*Soil:* The loam soil used in this experiment came from a garden on the National University of Ireland Maynooth campus and had the following composition by weight: 33% sand, 48% silt, 19% clay; 22.5% organic matter (assessed by the Teagasc Soil Laboratory, Johnstown Castle, Ireland). The soil was air-dried on the laboratory bench before use. Tap water was then added to bring it to the desired moisture content. Moisture content of the adjusted soil was measured using an MA30 moisture analyzer (Sartorius AG, Goettingen, Germany), and pF was determined as described by Hamblin (1981).

*Persistence in soil:* Plastic beakers (250-ml with snap-on

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lid; Wilscanco Plastics Ltd., Dungannon, Co. Tyrone, Ireland) containing 120 g soil each were inoculated with 16,000 IJ, applied in 2 ml of tap water. Controls received tap water alone. After inoculation, soil moisture was 18% (''dry'', pF 3.3) or 30% (''moist'', pF 1.7) by weight. Beakers, arranged in randomized complete blocks, were incubated in the dark at 20 °C. They were surrounded by a row of beakers containing the same soil as the experimental beakers to act as a buffer. Soil moisture was corrected weekly by adding water to each experimental beaker, as necessary, to maintain a previously determined weight. At intervals, three blocks of moist soil and three of dry soil were randomly chosen. There were thus three replicates of each treatment. The moisture content of the soil of each beaker was adjusted to 24% (pF 2.7) by mixing with an equal amount of uninfested soil of higher or lower water content. This was done to provide equal moisture conditions for all treatments in the infectivity tests. The adjusted soil was then divided into two halves of equal weight. One half of the soil was used for the determination of infectivity by baiting with *T. molitor,* while IJ were extracted from the other half by centrifugal flotation.

*Infectivity in soil:* Forty *T. molitor* larvae were lightly mixed into each soil sample to be baited and incubated for 4 days at 20 °C in the dark. Numbers of insects dead, alive, and parasitized (as evidenced by characteristic red color) were recorded 3 days after removal from the soil, during which time they were maintained at 20 °C.

*Extraction of nematodes from soil:* Nematodes were extracted from soil using centrifugal flotation (CF) (Jenkins, 1964), modified slightly (Hass et al., 1999). Nematodes remaining in the water supernatant, as well as those in the sucrose supernatant, were counted. Survival was expressed as a percentage of the number of IJ extracted on the first assessment date. Indigenous nematodes occurring in the soil samples were distinguished from *Heterorhabditis* IJ by size, developmental stage, and behavior (movement in tap water). The efficiency of the CF method for each of the four isolates was tested in a preliminary experiment in which freshly harvested (day-old) IJ were applied to soil (23% by weight soil moisture) and extracted immediately after application.

*Assessment of nematode optical density by image analysis densitometry:* The rate of utilization of reserves was as-

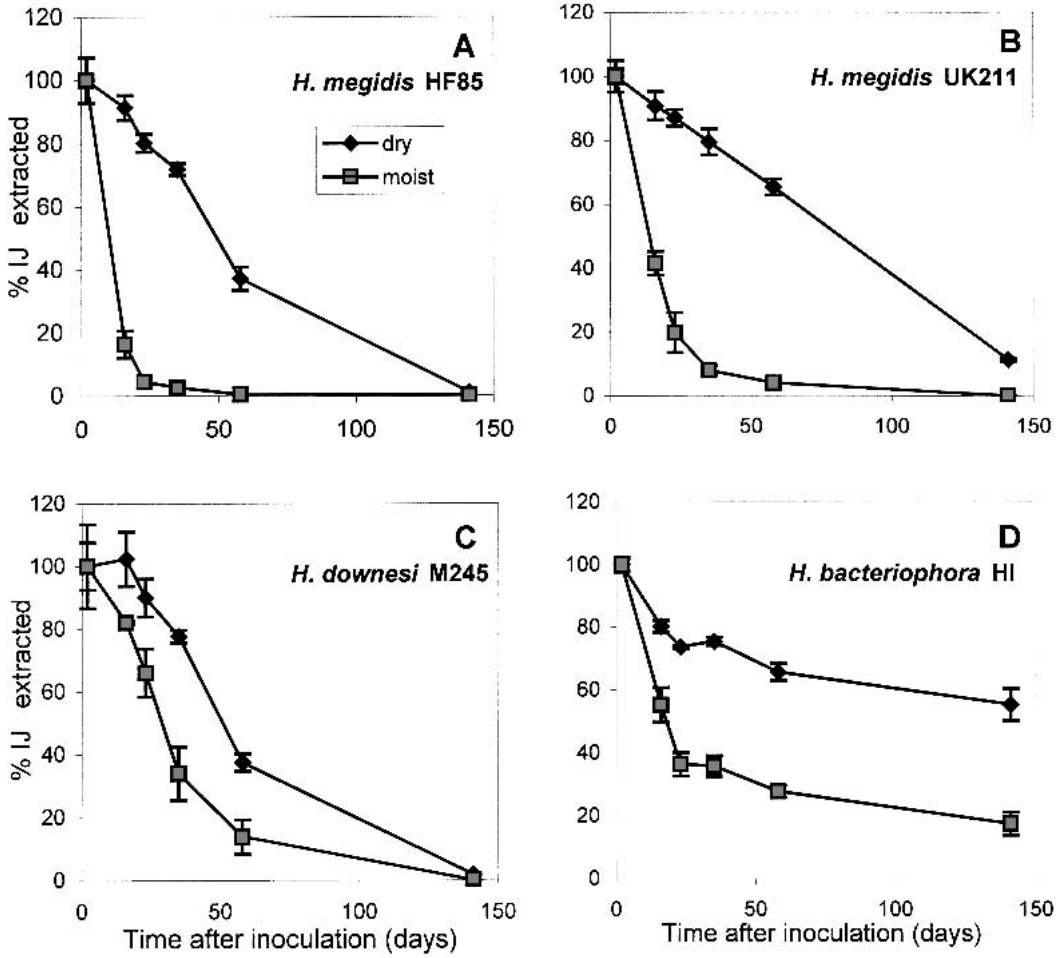


FIG. 1. Survival of four *Heterorhabditis* spp. isolates in moist (pF 1.7) and dry (pF 3.3) soil, measured as living IJ extracted by centrifugation flotation expressed as a percentage of the number extracted on day 2 (mean ± SE, $n = 3$). A) *H. megidis* HF85. B) *H. megidis* UK211. C) *H. downesi* M245. D) *H. bacteriophora* HI.

sessed as a decrease in optical density (OD) of IJ by image analysis densitometry (Fitters et al., 1997; Qiu and Bedding, 1999). The OD of live IJ extracted from soil on days 2, 16, 23, 35, and 141 was measured using a ''Magiscan'' image analyzer and GENIAS software (both from Applied Imaging, Sunderland, UK) following a method similar to that of Fitters et al. (1997). Images were captured by a video camera mounted on a Nikon Optiphot-2 microscope, using a ×10 objective. The area and integrated OD of each nematode IJ was measured. Optical density was divided by the area of the nematode to obtain more comparable values (OD/$\mu m^2$) for nematodes of different sizes. An average of 24 nematodes were measured per replicate soil sample.

*Statistical analysis:* Survival and infectivity data were subjected to probit analysis, with a Chi-square test for equality of slopes. Optical density data were analyzed by ANOVA. If significant overall treatment effects were found at $P < 0.05$, means were separated using Tukey's test. Correlation between parameters was tested using the Pearson product moment.

## Results

*Survival:* Survival declined over the 141-day period (Fig. 1). The rate of decline differed between isolates and was affected by soil moisture content, as evidenced by differing slopes of the regression lines of probit transformed data (Table 1). In dry soil, *H. bacteriophora* HI and *H. megidis* UK211 declined at a slower rate than the other two isolates; in moist soil, *H. bacteriophora* HI and *H. downesi* M245 were the isolates that declined at the slowest rate. In general, survival was greater in dry than in moist soil, but the difference between dry and moist soil was smaller for *H. downesi* M245 (Fig. 1C) than for each of the other three isolates. On day 141, the last assessment date, HI was the best surviving isolate at both moisture levels, with 18% survival in moist and 55% in dry soil (Fig. 1D), compared with 0% and less than 12% survival of the other three isolates in moist and dry soil, respectively.

Table 1. Survival of infective juveniles of four *Heterorhabditis* spp. isolates in dry (pF 3.3) and moist (pF 1.7) soil.

| Soil | Nematode | Slope[a] (SE) | LT50 (95% fiducial limits) days |
|---|---|---|---|
| Dry | *H. megidis* UK211 | –0.020 a (0.0001) | 79.28 (78.64–79.92) |
| | *H. megidis* HF85 | –0.032 b (0.0003) | 51.77 (51.30–52.26) |
| | *H. downesi* M245 | –0.032 b (0.0003) | 56.59 (56.06–57.13) |
| | *H. bacteriophora* HI | –0.007 c (0.0000) | 140.91 (138.54–143.39) |
| Moist | *H. megidis* UK211 | –0.071 d (0.0005) | 16.34 (16.17–16.51) |
| | *H. megidis* HF85 | –0.124 e (0.0009) | 11.05 (10.92–11.18) |
| | *H. downesi* M245 | –0.046 f (0.0004) | 31.36 (31.09–31.62) |
| | *H. bacteriophora* HI | –0.012 g (0.0001) | 32.55 (31.85–33.24) |

[a] Values followed by the same letter are not significantly different at $P < 0.05$.

The extraction efficiency for the four *Heterorhabditis* isolates ranged from 62% to 69%. Differences between isolates were not significant ($P > 0.05$).

*Infectivity:* In general, infectivity (Fig. 2) showed the same trends as survival (Fig. 1). Soil moisture did not affect the infectivity of *H. downesi* M245 (slope and LT50 equal in dry and moist soil); for each of the other three isolates, infectivity declined at a faster rate in moist than in dry soil, as evidenced by the steeper slope of the regression on probit transformed data (Table 2). There were differences in the rate of decline of infectivity among isolates both in moist and in dry soil. In dry soil, *H. megidis* UK211 and *H. bacteriophora* declined at a slower rate than *H. megidis* HF85 and *H. downesi*. Comparing the two isolates where infectivity declined more slowly, the LT50 of *H. bacteriophora* was significantly higher than that of UK211, based on non-overlap of fiducial limits (Table 2). In moist soil, the infectivity of *H. downesi* and *H. bacteriophora* declined most slowly and *H. downesi* had higher LT50 than *H. bacteriophora*. The most striking difference between isolates was in dry soil on day 141, when the rank order was HI > UK211 > (HF85 and M245) (Fig. 2).

*Optical density:* Storage in soil affected the OD of the isolates differently. In dry soil, the two *H. megidis* isolates and *H. downesi* each underwent a reduction in OD in dry soil over time, but *H. bacteriophora* did not (Fig. 3). At the start of the experiment the isolates did not differ in OD (day 2: $P > 0.05$ for both wet and dry soil). *Heterorhabditis bacteriophora* HI was darker ($P < 0.05$) than the other three isolates on day 141 in dry soil and on day 35 in moist soil.

A more biologically meaningful way of expressing the decline in OD is as a proportion of density or body contents available for use. When stored in water IJ become visibly paler and eventually die when little or no evidence of stored reserves is visible. Infective juveniles were stored in water until some of the population was dying; the most transparent individuals still alive at the time when others were apparently dying of starvation were selected for measurement. These starved IJ had an OD of approximately 0.2 OD units/$\mu m^2$ (unpub. data). Optical density levels higher than this can be considered ''available OD,'' representing material that can be depleted during storage. There was no change in mean available OD for *H. bacteriophora* HI in moist soil ($P > 0.05$); for other treatments there was a reduction ($P < 0.05$) ranging from 6% for HI in dry soil to 51% for *H. downesi* M245 in dry soil. This indicates that surviving IJ still had ample reserves even when most of the population had died.

In both soil types, *H. bacteriophora* HI underwent little

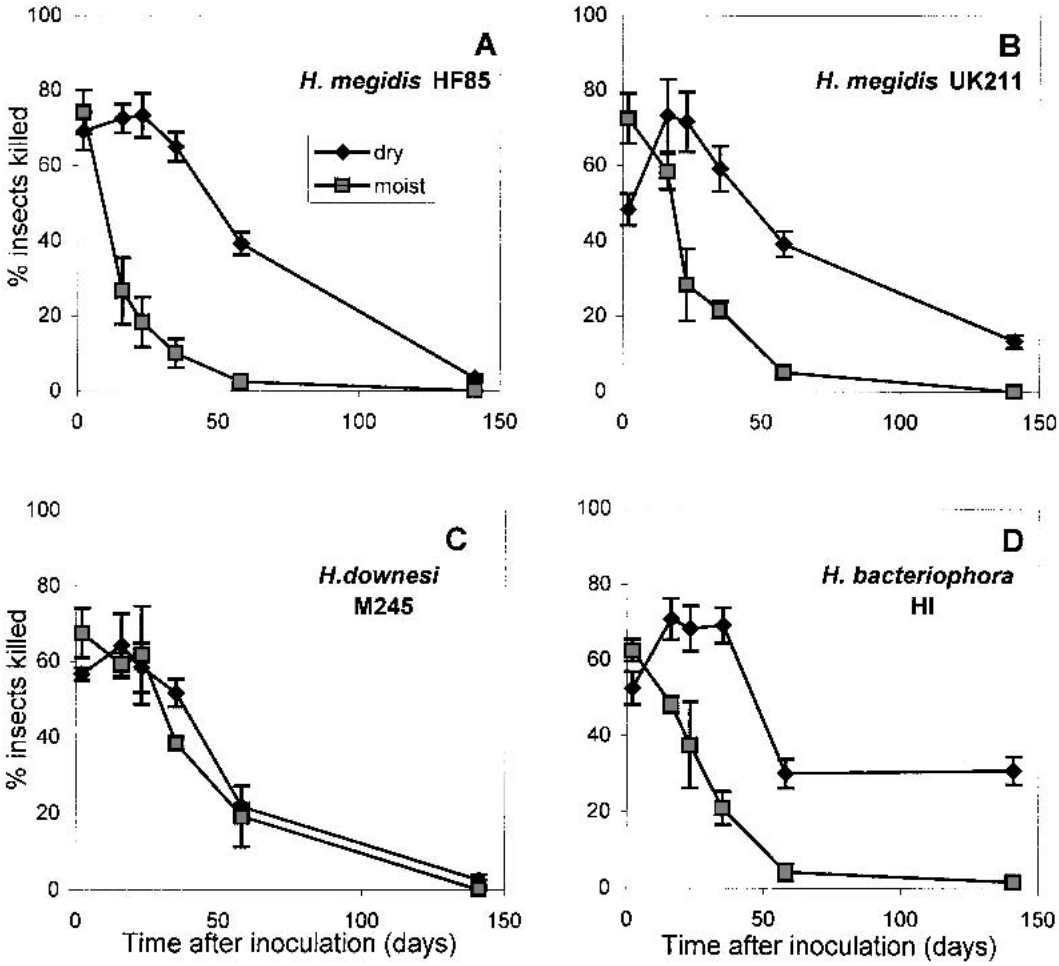


FIG. 2. Mortality (mean ± SE, $n = 3$) of bait insects (*Tenebrio molitor* larvae) in moist (pF 1.7) and dry (pF 3.3) soil inoculated on day 0 with IJ of four *Heterorhabditis* spp. isolates. A) *H. megidis* HF85. B) *H. megidis* UK211. C) *H. downesi* M245. D) *H. bacteriophora* HI.

TABLE 2. Persistence of infectivity of four *Heterorhabditis* spp. isolates in dry (pF 3.3) and moist (pF 1.7) soil.

| Soil | Nematode | Slope[a] (SE) | LT50[b] (95% fiducial limits) days |
|---|---|---|---|
| Dry | *H. megidis* UK211 | –0.011 a (0.001) | 45.45 (36.63–54.74) |
| | *H. megidis* HF85 | –0.018 b (0.002) | 46.79 (41.07–53.19) |
| | *H. downesi* M245 | –0.017 b (0.002) | 27.32 (21.27–33.06) |
| | *H. bacteriophora* HI | –0.007 a (0.001) | 58.44 (45.16–74.37) |
| Moist | *H. megidis* UK211 | –0.042 c (0.004) | 16.09 (13.00–18.82) |
| | *H. megidis* HF85 | –0.052 c (0.005) | 9.10 (5.88–11.74) |
| | *H. downesi* M245 | –0.025 b (0.003) | 25.43 (21.20–29.57) |
| | *H. bacteriophora* HI | –0.026 b (0.003) | 10.19 (4.36–14.74) |

[a] Values followed by the same letter are not significantly different at $P < 0.05$.
[b] LT50: the time at which 50% of insects used to bait the soil were killed.

reduction in OD (Fig. 3) and had the greatest survival of the four isolates (Fig. 1). Association between survival and OD was tested using day 35 data for moist soil and day 141 data for dry soil. For each soil there was correlation between survival of isolates and the extent to which their OD was depleted (moist soil: $r^2 = 0.88$, $P = 0.04$; dry soil: $r^2 = 0.82$; $P = 0.06$). This indicates that isolates with low survival also had lower OD. There was no evidence of an association between survival rates on the same days and starting levels of OD in either moist ($r^2 = 0.0$; $P = 0.62$) or dry ($r^2 = 0.34$; $P = 0.25$) soil.

## DISCUSSION

Infective juveniles of three of the four *Heterorhabditis* isolates underwent a decrease in OD in soil, indicating that they were using up their stored lipid reserves (Fitters et al., 1997; Qiu and Bedding, 1999). The maximum decrease in ''available density,'' representing utilizable reserves, was 51% recorded for *H. downesi* M245 after 141 days in dry soil. Depletion of lipid reserves below a critical level (typically 50% to 65% initial levels) results in a reduction in motility and infective potential

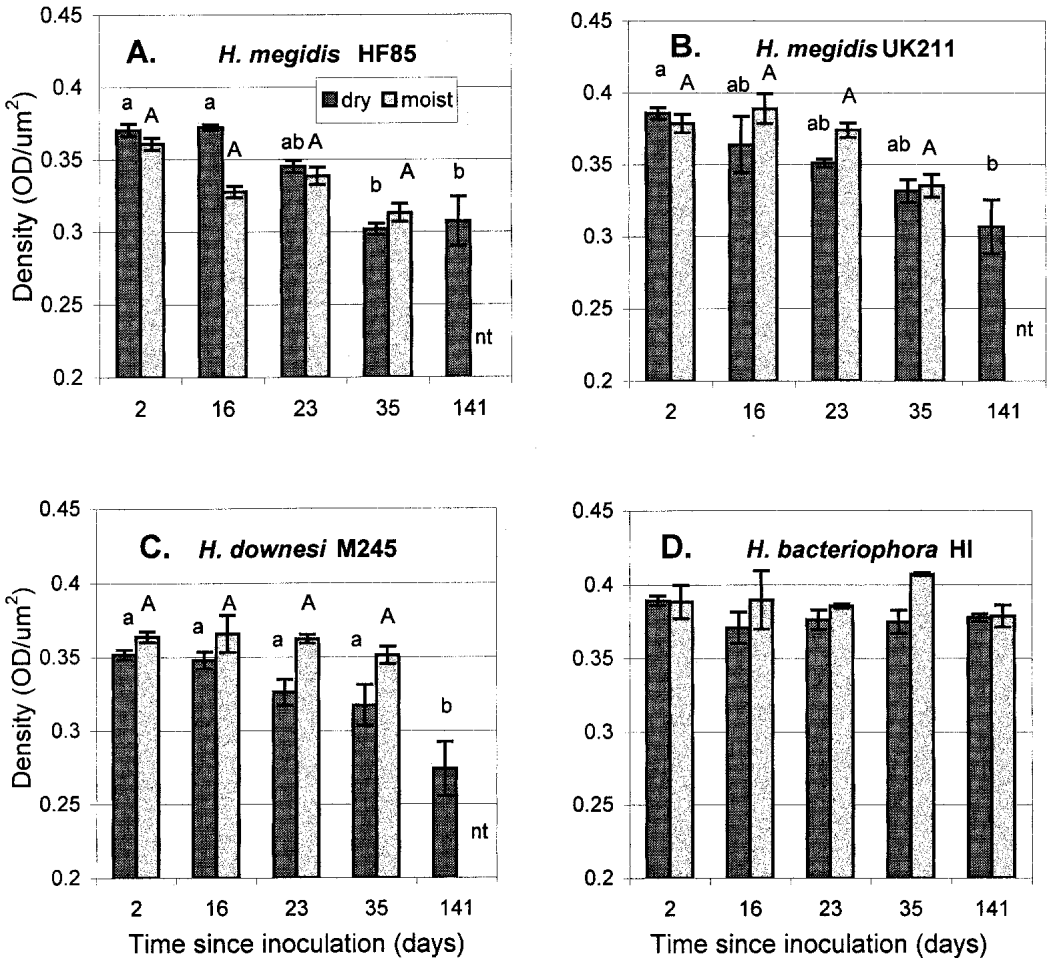


FIG. 3. Optical density/unit area (OD units/$\mu m^2$, mean ± SE, $n = 3$) of IJ of four *Heterorhabditis* spp. isolates after storage in moist (pF 1.7) and dry (pF 3.3) soil. A) *H. megidis* BF85. B) *H. megidis* UK211. C) *H. downesi* M245. D) *H. bacteriophora* HI. Within soil type, bars accompanied by the same letter (or none) are not significantly different ($P < 0.05$, Tukey's test).

of plant-parasitic nematodes (Robinson et al., 1987; Storey, 1984; van Gundy et al., 1967); for three *Steinernema* species in water there was a gradual decline in infectivity with lipid depletion from the start (Patel et al., 1997). The loss of lipid and other reserves may have contributed to the decline in infectivity of *Heterorhabditis* in the present experiment though, for the most part, survival and infectivity declined at a similar rate, suggesting that diminishing infectivity was largely due to death of IJ rather than deteriorating quality of surviving IJ.

Loss of 50% or more of available reserves has been recorded for nematodes stored in water, without appreciable mortality (Fitters, 1999; Grewal, 2000b; Qiu and Bedding, 2000). While lipid depletion may be an important cause of nematode mortality in water, data from the present experiment do not support the hypothesis that it is a direct cause of mortality for *Heterorhabditis* in soil. This is especially true for *H. bacteriophora* HI, where the mean OD of IJ extracted from moist soil showed no significant change from day 2 to day 141, while the percentage of IJ surviving dropped by 88% over this period. It is theoretically possible that a subset of the population became quiescent, retaining reserves and surviving to the end of the experiment, while a second subset depleted its reserves and died. However, this latter subset could represent only a small proportion of those that died: OD measurements made at intervals over a time when substantial numbers of IJ were depleting their reserves toward starvation level must necessarily result in a detectable change in average OD.

If, as it appears, starvation is not the primary cause of mortality for any *Heterorhabditis* isolate in this experiment, then it follows that differences between isolates in rate of utilization of reserves cannot directly account for their differing mortality rates in soil. Nevertheless, there was significant correlation between depletion of reserves and the mortality rates of the various isolates. For example, *H. bacteriophora* HI, which survived at highest numbers, was also the isolate that underwent the least change in reserves. So, if depletion of energy reserves was not *directly* responsible for the rapid decline in IJ numbers, what caused their death, and why is

conservation of reserves of some isolates associated with their greater survival in soil? It should be noted that survival was affected by soil moisture, which raises the possibility of differential weighting of mortality factors in the two soil moisture regimes. All four *Heterorhabditis* isolates survived better in dry (pF 3.3) than in moist (pF 1.7) soil. Similarly, *Steinernema carpocapsae* and *S. glaseri* inoculated into a sandy loam soil survived better at low soil moistures than at higher (Kung et al., 1991). We consider three possible reasons why nematodes may survive better in dry than in moist soil: (i) dry soil provides less opportunity for movement (Wallace, 1958) and hence promotes the conservation of reserves, (ii) survival may be lower in moist soil because of the reduced oxygen content (Kung et al., 1990), and (iii) the activity of nematode antagonists is encouraged in moist soils.

We can probably discount the first two of these as an explanation for the difference in survival of *Heterorhabditis* in moist and dry soil in our experiment. Soil at pF 3.3, the moisture content of the dry soil used in our experiments, should permit movement of entomopathogenic nematode IJ (Hass et al., 2001; Koppenhöfer et al., 1995; Molyneux and Bedding, 1984). Indeed, there was a trend in our experiment for greater rather than lesser utilization of reserves in dry soil (Fig. 3). It is also unlikely that at pF 1.7, the moisture content of the moist soil used here, oxygen levels were so low as to cause nematode mortality. However, the tendency for greater retention of body contents by IJ in moist soil is consistent with the hypothesis that lower oxygen content caused a reduction in motility and (or) metabolism of the nematodes, without contributing to their death. Van Gundy et al. (1967) also found that the body contents of juvenile *Meloidogyne javanica* and *Tylenchulus semipenetrans* were expended more slowly in moist than in dry soils.

Because non-sterile soil was used in the experiment, one possibility is that moist conditions favored the activity of nematode antagonists. Potential predators (mites and collembolans) were occasionally observed, but at such low numbers that they are unlikely to have been able to effect, for example, the dramatic decline in numbers of IJ in moist soil between days 2 and 16. However, microbial antagonists may considerably influence nematode persistence in natural soil, with effects apparent within 14 days (Gibb and Buhler, 1998; Ishibashi and Kondo, 1986).

If biotic factors are a cause of mortality, then differences between isolates in susceptibility to pathogens could have contributed to differences in survival. Such differences might include motility, retention of the J2 cuticle (sheath), or susceptibility to adhesion by pathogen propagules (Timper et al., 1991). More active nematodes have a greater probability of encountering soil pathogens, such as fungal spores, than less active nematodes and are also likely to use their reserves more quickly. Thus, an observed relationship between rates of lipid depletion and survival of nematode isolates in the presence of antagonists might be mediated by the common factor of motility. There may be an even more direct relationship between energy reserves and tolerance to abiotic stress. Energy reserves may affect stress tolerance; for example, starved *Longidorus elongatus* were more susceptible to nematicidal chemicals than well-fed ones (Alphey, 1983). A nematode isolate that used its energy reserves at a slower rate might be expected to tolerate stressful conditions better and hence survive longer in soil.

The data presented here confirm previous findings (Griffin et al., 1994; Hass et al., 2001) that *H. bacteriophora* is a more persistent nematode than either *H. megidis* NWE type or *H. downesi,* and also show that it uses its reserves at a slower rate. Differences in longevity between species of *Steinernema* correlated well with initial lipid content and with the rate of lipid utilization (Grewal, 2000a). Typically, large nematode species survive starvation longer than small species with the same percentage lipid content (Atkinson, 1980). Patel et al. (1997) found that species of *Steinernema* with relatively small IJ such as *S. carpocapsae* and *S. riobravis* used their lipid faster than those with larger IJ such as *S. feltiae* and *S. glaseri*. This relationship does not hold for *Heterorhabditis* isolates in soil; IJ of *H. bacteriophora* are shorter (typically <600 μm) than those of the other two species (>700 μm). Selvan et al. (1993) suggested that interspecific differences in survival of entomopathogenic nematodes in water could be accounted for by levels of active movement. Isolates of *H. megidis* and *H. downesi* were more active migrators than *H. bacteriophora* HI in sand columns at 20 °C (Westerman, 1995) and also displayed a higher level of activity (measured in waves/minute) in tap water (Griffin, unpubl.) Of course, differences in composition of energy reserves or in basal metabolic rates may also play a role in differential rates of energy conservation between isolates.

Overall, natural enemies and failure to cope with immediate abiotic stresses may explain early mortality when IJ are added to soil. The IJ of isolates that persist better in the long term must cope with the physical environment and escape natural enemies through behavioral or physiological resistance mechanisms. *Heterorhabditis bacteriophora* HI, the most persistent isolate in this experiment, also had the slowest rate of utilization of reserves, and we suggest that reduced activity, as part of an energy-conserving strategy, also results in avoidance of some antagonists. It is also likely that persistent isolates such as HI have additional resistance mechanisms. In comparison to *H. bacteriophora* HI and the two *H. megidis* isolates, *H. downesi* M245 was relatively unaffected by soil moisture and survived relatively well in moist soil. It is tempting to believe that *H. downesi,* or at least this particular isolate, which originated on the north coast of Ireland, is adapted to moist

oceanic conditions. Survival in moist soil was initially superior even to that of *H. bacteriophora* HI, though it did not display the long-term persistence of that isolate, and the two isolates may have different, complementary resistance mechanisms.

One of the major constraints on the commercialization of entomopathogenic nematodes is the poor shelf-life of formulated product. A more direct dependence of survival on the depletion of reserves might be expected under the more sterile conditions of formulation, as compared to the non-sterile soil of our experiment. A slow rate of energy utilization such as that displayed by *H. bacteriophora* HI is a desirable trait for shelf-life, though undoubtedly there must be a trade-off in other fitness parameters. We are still far from understanding what the trade-offs are in native entomopathogenic nematodes, and to what extent it is possible to combine desirable traits such as energy conservation and high host-finding ability in the one nematode strain.

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