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Environmental tolerances and the dispersal of *Heterorhabditis* : survival and infectivity of European *Heterorhabditis* following prolonged immersion in seawater

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Summary – Infective juveniles (IJs) of six European isolates of *Heterorhabditis* were stored for up to 19 weeks at 20 °C in either seawater or distilled water. Their survival and infectivity for *Galleria mellonella* larvae was assessed at intervals. The isolates tested were of three biological groups : *H. bacteriophora,* the North-West European Group and the Irish Group. Each of the isolates survived at least 19 weeks in seawater and was infective to *G. mellonella* after that time. It is argued that transport of infective juveniles by sea currents is a plausible means of dispersal of *Heterorhabditis* between land masses, or between coastal sites on a single landmass. Differences between isolates were identified, especially in distilled water. *H. bacteriophora* survived best and the Irish Group worst. The infectivity of the Irish and NWE isolates declined progressively with time of storage, while that of *H. bacteriophora* initially increased and then returned to the starting level. The Irish and NWE isolates survived better and remained infective for longer when stored in seawater then in distilled water.

Résumé – ***Tolérances environnementales et dispersion d'*Heterorhabditis : *survie et pouvoir infestant d'isolats européens d'*Heterorhabditis *après immersion prolongée dans l'eau de mer*** – Des juvéniles infestants provenant de six isolats européens d'*Heterorhabditis* ont été maintenus pendant des périodes allant jusqu'à 19 semaines à 20 °C dans l'eau de mer ou l'eau distillée. Leur survie et leur pouvoir infestant vis-à-vis des larves de *Galleria mellonella* ont été périodiquement évalués. Les isolats testés appartenaient à trois groupes différents : *H. bacteriophora,* le groupe de l'Europe du Nord-Ouest et le groupe irlandais. Chacun des isolats survit au moins 19 semaines dans l'eau de mer et conserve son pouvoir infestant envers *G. mellonella* après cette période. L'hypothèse est avancée que le transport des juvéniles infestants par les courants marins pourrait être un moyen de dispersion d'*Heterorhabditis* entre les terres émergées (continents ou îles) ou entre les sites côtiers d'une même région. Des différences de comportement entre isolats ont été observées, en particulier dans l'eau distillée. La survie d'*H. bacteriophora* est la meilleure, celle du groupe irlandais la moins bonne. Le pouvoir infestant des isolats des groupes irlandais et de l'Europe du N.O. diminue progressivement pendant le stockage, tandis que celui d'*H. bacteriophora* augmente dans un premier temps pour retrouver ensuite son niveau initial.

Key-words : *Heterorhabditis,* entomopathogenic nematode, dispersal, biogeography, tolerance, seawater, survival, physiology.

For soil dwelling nematodes, human agency is considered to be the most effective means of dispersal (Ferris *et al.,* 1976), and it has been suggested that entomopathogenic nematodes have been spread between continents and to remote islands by this means, either in ships ballast or with exotic plants (Akhurst & Bedding, 1986; Hara *et al.,* 1991). Animals other than humans, particularly insects (Timper *et al.,* 1988) may also serve as agents of dispersal.

In Ireland and Britain, heterorhabditids are common in sandy coastal habitats (Griffin *et al.,* 1994), but apparently absent or rare in other habitats (Blackshaw, 1988; Hominick & Briscoe, 1990; Griffin *et al.,* 1991; Boag *et al.,* 1992) and thus have a patchy " necklace " distribution around the coast. It has been suggested (Hominick, 1990) that populations of entomopathogenic nematodes may undergo periodic extinctions followed by re-introduction from neighbouring sites. In this situation, neighbouring sites are separated by areas of inhospitable terrain, and so again jump dispersal – by human, animal, or other agency – must be involved.

We suggest that the sea may serve as a carrier of heterorhabditids from one isolated coastal habitat to another. Transport by sea currents might also account for dispersal of heterorhabditids to islands such as Ireland or Britain or Hawaii, or between continents. For transport by sea currents to be an effective means of dispersal, the infective juveniles (IJs) would need to survive immersion in seawater and to remain infective to insects. Using strains of three different groups (Smits *et al.,* 1991; Dix *et al.,* 1992), *H. bacteriophora,* the North-West European Group and the Irish Group, we test whether European *Heterorhabditis* species have this capacity.

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Materials and methods

The identity and origin of the six *Heterorhabditis* isolates used is given in Table 1. HF85 was obtained from Ir Paula Westerman, Friesland College of Agriculture, The Netherlands. UK211 was from Dr. W. M. Hominick, Imperial College London. The other isolates were from our own collections. Nematodes were cultured at 20 °C in late instar larvae of the wax-moth *Galleria mellonella*. They were harvested in modified White traps, washed by sedimentation in three changes of tapwater and used within 10 days of harvesting.

Table 1. *Identity and origin of the* Heterorhabditis *isolates used in the study.*

Code	Species or Group	Origin
EU222	*H. bacteriophora*	Hungary
EU185	*H. bacteriophora*	Hungary
UK211	North-west European	South coast of England
HF85	North-west European	Flevopolder, Netherlands
W48	Irish	South coast of Wales
M170	Irish	North-west coast of Ireland

Suspensions of infective juveniles (100/ml) were prepared in seawater (from Sandycove, Co. Dublin) or distilled water. Aliquots of 8 ml were stored in 5 cm diam Petri dishes at 20 °C for up to 19 weeks. There were three replicate dishes for each treatment and assessment date. At intervals, the numbers of live nematodes were counted, and the infectivity of the surviving nematodes for *Galleria mellonella* larvae was assessed. In addition, some of the nematodes which had been stored in seawater were washed three times by sedimentation through a column of distilled water, and incubated in distilled water at 20 °C for 4 days prior to testing infectivity.

Infectivity was tested in sand (heat sterilized silver sand moistened with 8 % (w/w) tapwater). A late instar *G. mellonella* larva was placed in a 2.5 cm diam. Petri dish and covered with moist sand. Ten nematodes were picked out (by reference to preselected locations in a gridded dish) and added to the sand in a small volume of water (*ca* 40 µl). A total of fifteen insects was exposed per treatment (five for each replicate dish of water). The insects were incubated at 20 °C for 4 days, washed in tapwater and patted dry with paper towelling to remove nematodes adhering to the cuticle. They were returned to 20 °C and dissected after a further 2-3 days. The number of first generation females was taken as representative of the number of infective juveniles that had entered.

Data were analysed by Kruskall Wallace " ANOVA " followed by pairwise comparisons (Mann Whitney U Test).

Results

Survival

All of the six isolates survived at least 19 weeks immersion in seawater (Fig. 1). *H. bacteriophora* Eu185 survived less well in seawater than in distilled (Fig. 1 B) while the other five isolates survived better in seawater than in distilled (Fig. 1 A, C-F).

Only three of the isolates (Eu222, Eu185 & UK211; Fig. 1 A-C) survived the full 19-week period in distilled water. After 15 weeks, 80 % of Eu185, and at least 60 % of both Eu222 and UK211, were still alive; differences between these three isolates were not significant after either 15 or 19 weeks. By contrast, the survival of HF85 and of the Irish Group isolates M170 and W48 declined rapidly in distilled water, with few or none remaining alive after 15 weeks (Fig. 1 D-F). In seawater, differences between isolates were less marked. There was a significant difference ($P < 0.05$) between all six isolates after 1, 5 and 19 weeks, but in post-hoc tests between pairs of isolates, P was never less than 0.0495.

Infective juveniles in seawater were observed to move more slowly than those in distilled water; and they retained their dark appearance for longer.

Infectivity

After 19 weeks in seawater, surviving IJs of each isolate killed at least 50 % of the test insects (Table 2). When infective juveniles that had been stored in seawater were transferred to distilled water (washed) for four days prior to testing, their infectivity tended to improve (Table 2, Fig. 2). This is more readily seen from the results of the dissections presented in Figure 2. For each of the Irish isolates (Fig. 2 E, F) there was a significant ($P < 0.05$) difference between washed and unwashed treatments following 1, 8 and 15 weeks in seawater. A similar, significant, improvement is evident for HF85 after 8 and 15 weeks (Fig. 2 D). After 19 weeks there was either no difference, or there was evidence of a decline in infectivity following transfer of HF85 or the Irish isolates. In the case of UK211, washing effected an improvement in infectivity after 19 weeks (Fig. 2 C). There is also some indication that washing improved the performance of seawater-stored *H. bacteriophora,* during the earlier weeks of storage, but the difference between washed and unwashed treatments was not significant at any observation date (Fig. 2 A, B).

When stored in distilled water, the infectivity of *H. bacteriophora,* which was initially low, increased with time of storage, reaching a maximum after either 4-8 weeks (Eu222, Fig. 2 A) or 15 weeks (Eu185, Fig. 2 B). When stored in seawater, infectivity did not increase over time, and in EU222 there was a drop in week 15. The infectivity of the two NWE and of the two Irish isolates declined progressively during storage in distilled water (Fig. 2 C-F), though the decline was less rapid in the case of UK211 (Fig. 2 C). When these four

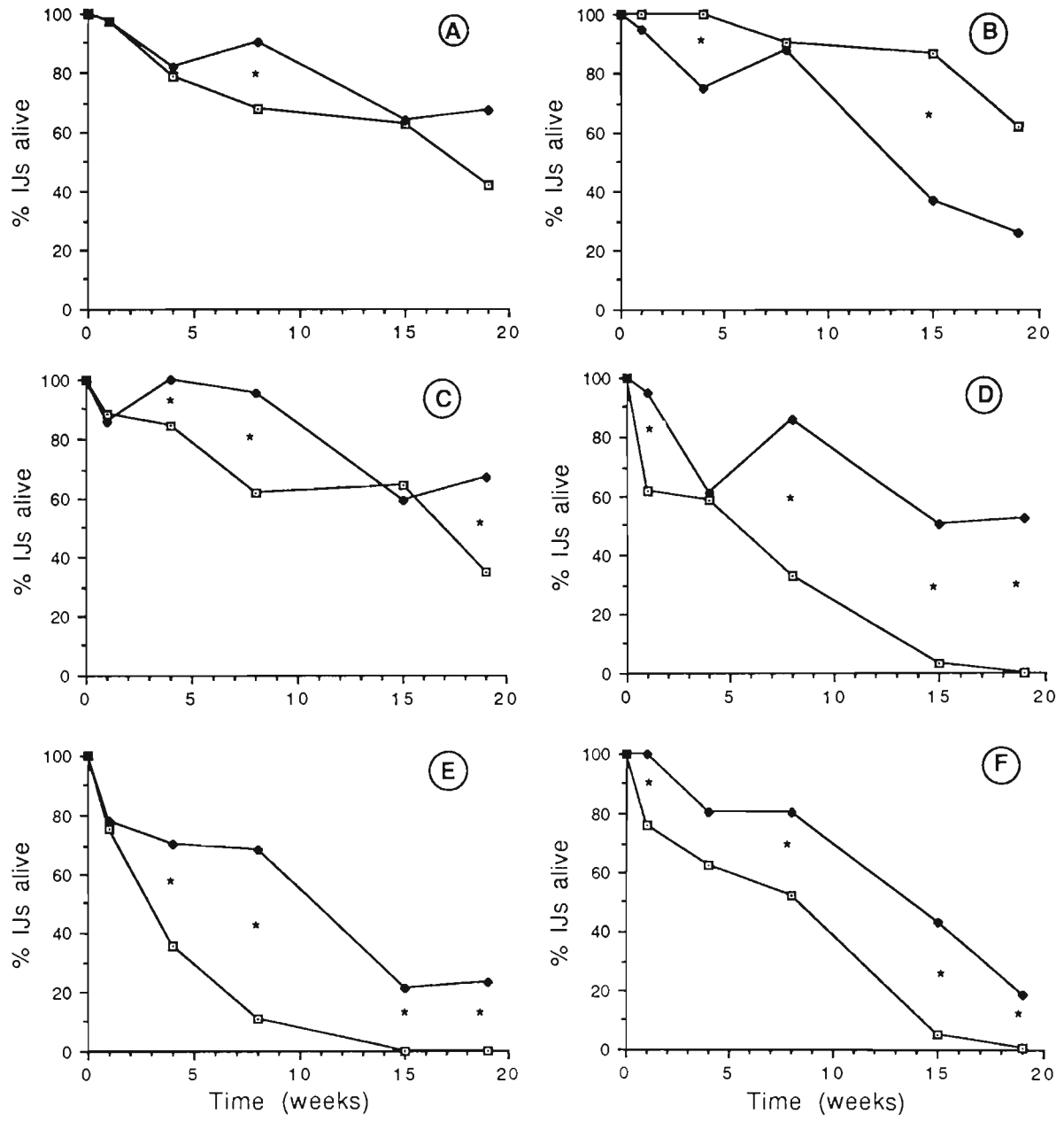

Fig. 1. *Percentage of infective juveniles of six* Heterorhabditis *isolates alive after various periods of storage in seawater or distilled water. A :* H. bacteriophora *Eu222; B :* H. bacteriophora *Eu185; C : NWE UK211; D : NWE HF85; E : Irish Group M170; F : Irish Group W48.* ◆ *= sea water;* □ *= distilled water. (Each point represents the mean of three replicates, with 800 nematodes in 8 ml of liquid. The asterisk indicates a significant difference between treatments; Mann Whitney U Test.)*

isolates were incubated in seawater, infectivity either remained constant or increased with up to 8 weeks storage, but declined thereafter (Fig. 2 C-F).

Differences in infectivity between the six isolates stored in distilled water were highly significant at all observation times (Kruskall Wallace " ANOVA ", $P \leq 0.001$; compare Fig. 2 A-F). After one week, the North-West European and Irish Group isolates performed similarly to each other but were much more infective than *H. bacteriophora;* at least three times as many infective juveniles established in *Galleria* larvae. However, after a further three weeks, *H. bacteriophora* Eu222 was one of the two most infective isolates (together with UK211) and each of these two isolates was

Table 2. *Percentage of* Galleria mellonella *larvae (n = 15) killed following exposure to infective juveniles (10 IJs/insect) of six* Heterorhabditis *isolates that had been stored for different periods of time in either distilled water or seawater.*

Isolate	Storage time (weeks)	Distilled water	Seawater	Seawater (washed)†
Eu222	1	66.7	66.7	93.3
	4	100.0	–	93.3
	8	100.0	73.3	93.3
	15	86.7	6.7	40.0
Eu222	19	80.0	93.3	93.3
Eu185	1	53.3	13.3	46.7
	4	86.7	–	46.7
	8	60.0	40.0	40.0
	15	100.0	13.3	0.0
	19	80.0	53.3	33.3
UK211	1	100.0	93.3	100.0
	4	100.0	–	100.0
	8	80.0	86.7	86.7
	15	73.3	6.7	100.0
	19	33.3	80.0	80.0
HF85	1	100.0	100.0	100.0
	4	80.0	–	100.0
	8	46.7	100.0	100.0
	15	–*	66.7	40.0
	19	–*	86.7	80.0
M170	1	93.3	100.0	100.0
	4	80.0	–	93.3
	8	46.7	93.3	100.0
	15	–*	60.0	100.0
	19	–*	93.3	86.7
W48	1	86.7	66.7	100.0
	4	66.7	–	100.0
	8	73.3	86.7	100.0
	15	20.0	20.0	100.0
	19	–*	63.3	60.0

† Nematodes that had been stored in seawater were washed in distilled water prior to testing.
– Not tested; –* Not tested as insufficient nematodes survived.

significantly different from all others. When the IJs were stored in seawater, the two *H. bacteriophora* isolates remained less infective than other isolates for eight weeks and this pattern was still very clear after fifteen weeks in the seawater (washed) treatment.

Discussion

Infective juveniles of each of the six *Heterorhabditis* isolates were infective to insects following prolonged immersion in seawater. This supports the hypothesis that transport by seawater, whether in suspension or in association with flotsam, is a plausible means of dispersal of the genus between land-masses or between isolated habitats along a coastline. Dispersal between isolated coastal habitats as in Ireland and Britain is probably more likely than dispersal between continents. Marine nematodes of the meiofauna are dispersed in this way (Platt & Warwick, 1988), and living nematodes are found in the plankton of coastal waters (Gerlach, 1977). In the laboratory, *Heterorhabditis* IJs settle very slowly to the bottom of vessels of seawater, though this medium is more buoyant than distilled water. At sea, however, turbulence could maintain them in suspension for longer, especially during stormy weather (Gerlach, 1977), a time when we would expect erosion of dunes to result in heterorhabditids entering the sea. Even if IJs do not remain in suspension, they might be washed to sea on rafts of land vegetation (Brown & Gibson, 1983) or become associated with floating material. Drifting seaweeds travel at rates of at least 20 km per day (Schwenke, 1971); nematodes that remained viable for 19 weeks could thus be dispersed over at least 2660 km. Most European ocean water remains below 20 °C, and European heterorhabditids survive longer at temperatures lower than 20 °C. Therefore, the experiments reported here probably underestimate the ability of heterorhabditids to survive under natural conditions. Some terrestrial invertebrates, including land molluscs, insects and spiders have been transported up to 3000 km either by swimming or on rafts (Gorman, 1979).

It is likely that the initial landfall of seaborne nematodes would be in relatively inhospitable terrain – the littoral zone and splash zone. However, flotsam (seaweed, sea-borne land-plants and timber) at the high tide line attracts an insect fauna of coleoptera and diptera which could provide opportunities for initial establishment or phoresis. Alternatively, nematodes could be carried by spray directly into the foredunes or other terrestrial vegetation. Froth may trap small particles, and we have observed spume blown in large quantities onto slopes with stable grass cover at a site where *Heterorhabditis* was detected. Establishment of *Heterorhabditis* following sea dispersal (or other rare dispersal events) is favoured by two features of its biology : a self-fertile hermaphrodite first generation and a high reproductive potential - several thousand IJs may result from the infection of a suitable host by a single IJ.

Although we did not test whether nematodes stored in seawater are infective in substrates of high osmotic potential, they were capable of infecting insects in sand when added to it in seawater. Infectivity was however improved by a recovery period in distilled water. On the basis of these findings, it is likely that *Heterorhabditis* would be infective immediately after landfall, but that the infectivity of IJs blown or washed ashore would improve following rainfall.

We suggest that migrating coastal dune material with rising sea levels following the last glaciation may have been effective means of returning entomopathogenic nematodes to warming northern latitudes. Indeed,

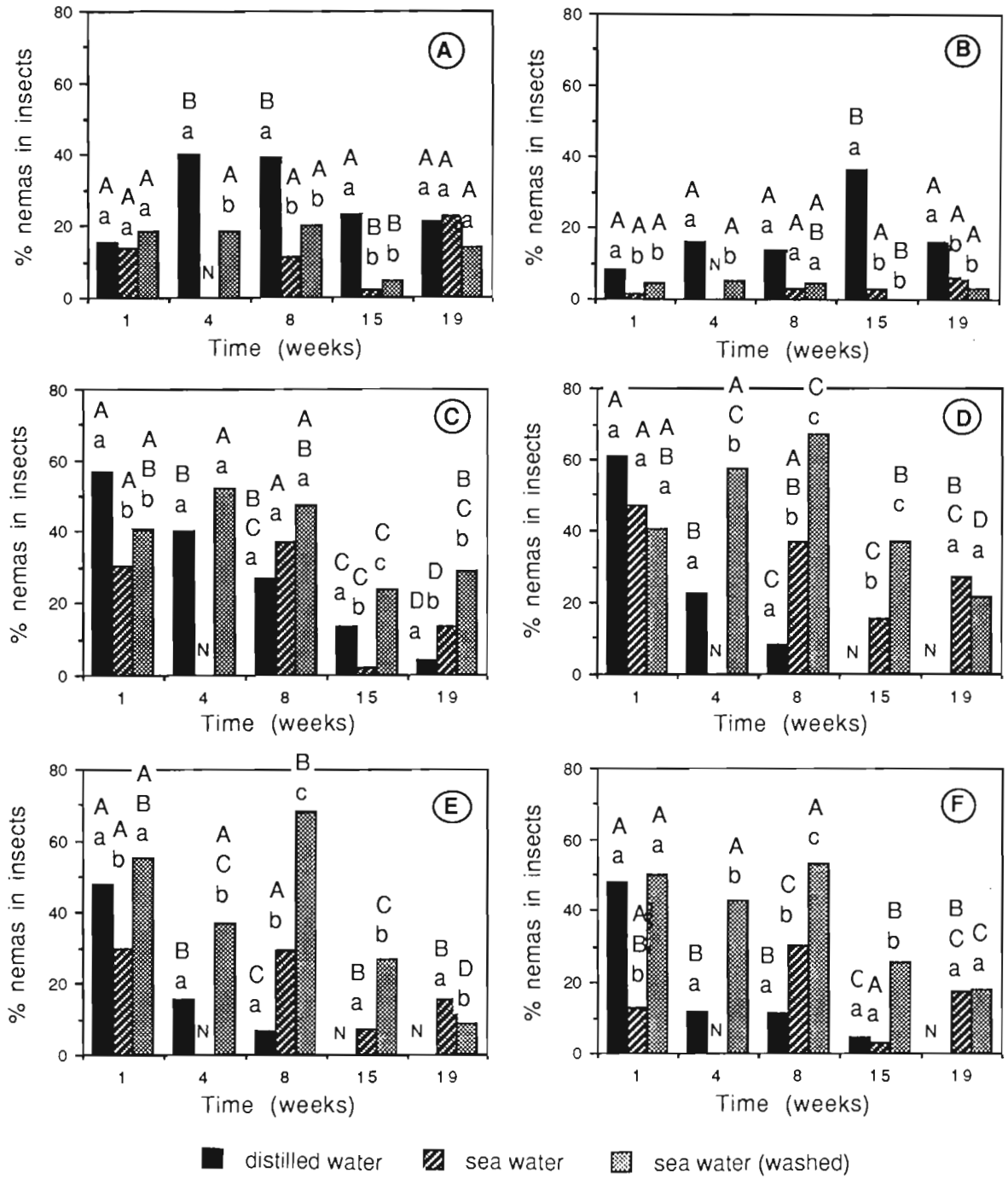

Fig. 2. *Infectivity (percentage of nematodes applied recovered in insects; ten nematodes per insect, replicated fifteen times) for* Galleria mellonella *larvae in moist sand, of* Heterorhabditis *IJs of six isolates following storage for different periods of time in distilled water or in seawater, with (washed) or without four days recovery in distilled water. A :* H. bacteriophora *Eu222; B :* H. bacteriophora *Eu185; C : NWE UK211; D : NWE HF85; E : Irish Group M170; F : Irish Group W48. (Lower case letters : comparison between treatments at any one assessment date; upper case letters : comparison between assessment dates for a given treatment; within each figure, columns accompanied by the same letter do not differ significantly. Kruskall Wallace " ANOVA " and pairwise Mann Whitney U Test;* $P < 0.05$. *N : not tested.)*

coastal types may have been dispersed widely during this period (among others). Current restrictions of their geographic ranges may therefore be related to their recent origin, or their tolerance of local environmental factors, or both, rather than to their failure to disperse.

Differences between isolates tended to coincide with taxonomic groupings. *H. bacteriophora* was clearly differentiated from the Irish Group. In distilled water, its infectivity increased during the period of the experiment; survival was either not markedly enhanced (Eu222), or was actually impaired (Eu185) by seawater, and the infectivity of nematodes stored in seawater was not greatly enhanced by transfer to distilled water. In contrast, members of the Irish group died more rapidly in distilled water, and the infectivity of surviving nematodes also declined rapidly; survival was enhanced in seawater; and the infectivity of nematodes stored in seawater increased significantly if they were first allowed to recover in distilled water for a few days. In general, the North-West European isolates performed similarly to the Irish Group, except that in distilled water UK211 survived as well as did *H. bacteriophora*. A rank order similar to this was reported by Hass (unpubl.) for the persistence of *Heterorhabditis* in clay loam soil and in tapwater; *H. bacteriophora* persisted longest, followed by the NWE Group, and there was overlap between the NWE and Irish Groups. The findings that the Irish Group and North-West European Group show some similarity in performance probably reflects the fact that they are more closely related to each other than to *H. bacteriophora* (Smits *et al.*, 1991; Joyce *et al.*, 1994), being even capable of limited hybridisation (Griffin *et al.*, 1994).

In both the present experiments and in those of Hass (unpubl.), UK211 survived longer than HF85. These two members of the North-West European Group originate on different sides of the English Channel. There is some corresponding evidence of intraspecific variation in *H. bacteriophora* from two sites in Hungary. In our experiments, Eu222 was more infective than Eu185, and this is supported by Addai (unpublished, this laboratory), who found that Eu222 was four times as infective (for *G. mellonella* larvae on filter paper) as Eu185. Intraspecific variation in the biological characteristics of heterorhabditids isolated from within the same small country has previously been demonstrated in both Irish (Hass *et al.*, 1992) and Dutch (Griffin *et al.*, 1989) nematodes.

Differences in the survival of nematode isolates might reflect either differences in rates of natural ageing including starvation, or differences in susceptibility to pathogens. Nematode infective juveniles utilise their stored food reserves, especially lipid, during storage (Storey, 1984). In tapwater at 20 °C, HF85 IJs utilise their stored food reserves rapidly; after 6 weeks less than 40 % remained alive and none of the remaining nematodes had ample fat reserves (Jung, 1991). It is likely that when stored in water, starvation is a major mortality factor for *Heterorhabditis* IJs. However, the inclusion of antibiotics enhanced the survival of *Heterorhabditis* sp. M145 in water (Finnegan *et al.*, 1992), suggesting the involvement of pathogens.

In seawater, most of the isolates (with the exception of Eu185) survived as well as, or better than, in distilled water. In seawater, the infective juveniles were observed to move more slowly and to remain dark for longer. While this was not quantified for *Heterorhabditis*, it is known that nematodes of varied taxonomic groupings either move more slowly or become completely inactive in hyperosmotic solutions (e.g. Croll, 1972; Reversat, 1981; Wharton *et al.*, 1983). In such solutions, respiration rates are lowered (Reversat, 1977) and nematodes utilise their stored food reserves more slowly (Croll, 1972; Reversat, 1981). We suggest that the IJs of the isolates which die more rapidly in distilled water (HF85, M170 and W48) utilise their lipid reserves more rapidly than the others, either due to a high basal metabolic rate or to high levels of motility, or both, and thus die early of starvation. This is supported by the finding that nematodes of the NWE and Irish Groups are more active than *H. bacteriophora* when tested in sand columns (Westerman, 1993). In seawater, where activity is inhibited, differences between the isolates were less marked.

The infectivity of nematodes stored in seawater was enhanced by transfer to distilled water prior to testing. However, for most of the isolates this was not the case after 19 weeks of storage. At this stage, transfer of the nematodes may have represented an additional physiological stress with which these aged nematodes could not cope. This is supported by the fact that in UK211, which appears to age more slowly, the increase in infectivity following transfer to distilled water was still observed after 19 weeks.

The infective juveniles of entomopathogenic nematodes are frequently stored in the laboratory in tapwater or distilled water (Woodring & Kaya, 1988). However, as shown here, these media may be suboptimal for some species of *Heterorhabditis*.

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