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1 Inundative pest control: how risky is it? A case study using entomopathogenic nematodes in a
2 forest ecosystem

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17

18 Abstract

19 Entomopathogenic nematodes (EPN) are globally important inundative biological control agents.
20 Their widespread use makes environmental risk assessment important, but very few
21 comprehensive post-application risk assessments have been conducted for EPN. We apply a
22 rigorous risk analysis procedure to the use of EPN applied in a forest ecosystem to suppress the
23 large pine weevil (*Hylobius abietis*). In this synthesis, we provide a quantitative evaluation of
24 five risk categories: a) establishment, b) dispersal, c) host range, d) direct non-target effects and
25 e) indirect non-target effects. A low level of risk was identified (35 – 51 out of a possible total of
26 125). Species exotic to the clear-fell forest ecosystem (*Steinernema carpocapsae* and
27 *Heterorhabditis downesi*) were accorded a lower overall risk status than native species and
28 strains (*Steinernema feltiae*), largely as a result of their shorter persistence in the target
29 environment. We conclude that EPN are a low risk viable alternative control for pine weevil
30 compared to the higher risk conventional control using pyrethroid insecticides.

31

32 Key Words: Risk assessment, Inundative biological control, Entomopathogenic nematodes, Pine
33 weevil, Forestry

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35

36 Inundative control with EPN and the potential associated risks

37 Entomopathogenic nematodes (EPN) are lethal insect pathogens that are commercially produced
38 as inundative control agents and used in various regions of the world against a variety of pests
39 (Kaya & Gaugler, 1993; Shapiro-Ilan et al., 2006; Grewal, 2012). There are two genera
40 (*Steinernema* Travassos, 1927 and *Heterorhabditis* Poinar, 1976: Nematoda: Rhabditidae), both
41 of which have global natural distributions (except Antarctica) and are used in biological control
42 (Kaya & Gaugler, 1993; Stuart et al., 2006). The free-living stage of the life cycle, the infective
43 juvenile (IJ), seeks out an insect host, invades it and releases entomopathogenic bacteria from its
44 gut that kill the insect within days (Kaya & Gaugler, 1993; Forst, 1997; Lewis et al., 2006). The
45 nematodes feed on the bacteria, reproduce and, typically after a period of two to three weeks, up
46 to several hundred thousand IJs leave the host cadaver to seek out new hosts. Since EPN have a
47 wide potential host range (Peters, 1996), can survive and reproduce in the field (Bathon, 1996;
48 Smits, 1996) and may disperse, including via phoresy (Eng et al., 2005; Campos-Herrera et al.,
49 2006) or within infected hosts (Downes & Griffin, 1996), they have the potential to cause
50 environmental impacts other than the intended pest reduction.

51 For assessing the risk of using inundative biological control organisms, van Lenteren et al.
52 (2003) identified five commonly agreed risk categories: host range, dispersal, establishment, and
53 direct and indirect non-target effects. To standardize risk assessment procedures, protocols for
54 assessing the risk of invertebrate biological control organisms in each of these categories have
55 been proposed (e.g. Babendreier et al., 2005; Clerq et al., 2011). A number of reviews
56 summarize the results of risk assessment studies on both classical and inundative biological
57 control organisms (e.g. Hokkanen and Lynch, 1995; Ehlers & Hokkanen, 1996; Barratt et al.,

58 2006 & 2010; van Lenteren et al., 2006). For classical and augmentative biological control Hajek
59 et al. (2016) have demonstrated widespread rather trivial effects of introductions and a few cases
60 of direct and indirect impacts at the population and community level mainly for older (pre 1950)
61 introductions. For EPN, extensive information exists relevant to the risk categories of
62 establishment (or persistence) (e.g. Wright et al., 1993; Shields et al., 1999; Koppenhofer &
63 Fuzy, 2006; Susurluk & Ehlers, 2008) and dispersal (e.g. Lacey et al., 1995; Jabbour &
64 Barbercheck, 2008), as well as host range (Peters, 1996). Direct and indirect non-target impacts
65 have received less attention (Bathon, 1996; Somasekhar et al., 2002; de Nardo et al., 2006;
66 Hodson et al., 2012). The available evidence indicates that EPN are generally safe, with little
67 environmental impact (Ehlers & Hokkanen, 1996), though there are very few examples of
68 comprehensive post-application risk assessments investigating multiple risk categories. The only
69 study that has so far investigated all five risk categories is that of van Lenteren et al. (2003) who
70 evaluated the risk of *Steinernema feltiae* (Filipjev, 1934) application in an open field. The
71 present case study summarises risk assessment research carried out on a range of EPN species
72 used to control the large pine weevil (*Hylobius abietis* L., 1758; Coleoptera: Curculionidae) and
73 evaluates the risk for strains that are both native and foreign to the target habitat using the
74 protocol of van Lenteren et al. (2003).

75 Large pine weevil control: Target pest, environment and control agents

76 The large pine weevil is a major forestry pest in 15 European countries, including Ireland and the
77 UK (Långström & Day, 2004). This insect threatens an estimated 3.4 million hectares of forests
78 and would cause up to € 140 million in annual damages if not controlled (Långström & Day,
79 2004). Larvae feed and develop under the bark of stumps and roots of recently dead conifers for

80 one or more years (Leather et al., 1999). Emerging adults feed on the bark of seedlings that are
81 planted to restock such sites, and this can result in up to 100 % of the seedlings being killed if the
82 pest is not controlled (Heritage et al., 1989; Leather et al., 1999; Petersson et al., 2005). Forestry
83 practices based on coniferous monoculture with clear-felling have favoured pine weevil, by
84 providing an optimum breeding habitat in stumps, and populations can be very high on clear-fell
85 sites (Leather et al., 1999).

86 EPN are currently being trialled in Ireland and the UK (including full operational application at
87 selected sites) to evaluate their potential as inundative control agents within an integrated
88 management strategy aimed at replacing pyrethroids (i.e. alpha-cypermethrin and cypermethrin)
89 currently used to control pine weevil (e.g. Brixey et al., 2006; Dillon et al., 2006; Williams et al.,
90 2013). To suppress weevil populations, EPN IJs in aqueous suspension are sprayed onto the soil
91 around the circumference of each tree stump on a site-wide level (recommended rate 3.5×10^6 IJs
92 per stump) to target the immature stages (Dillon et al., 2006). Several EPN species have been
93 tested: *Steinernema carpocapsae* (Weiser, 1955), *Steinernema kraussei* (Steiner, 1923) *S. feltiae*,
94 *Heterorhabditis downesi* Stock, Griffin and Burnell, 2002 and *Heterorhabditis megidis* Poinar,
95 Jackson and Klein, 1987 (Table 1) and all have shown potential to significantly reduce weevil
96 populations and/or seedling damage (Brixey et al., 2006; Dillon et al., 2006; Torr et al., 2007;
97 Williams et al., 2013). *Steinernema carpocapsae* is currently the main species in use due to its
98 competitive cost and amenability to mass production, though other species (especially *H.*
99 *downesi*) have shown better field efficacy.

100 Natural distribution of entomopathogenic nematode species used for pine weevil control

101 Organisms exotic to a particular environment may pose risks that differ in quality and scale from
102 those of indigenous organisms (Simberloff & Stiling, 1996; van Lenteren et al., 2003; Clerq et
103 al., 2011; van Lenteren, 2012). Ehlers and Hokkanen (1996) recommended that, unlike the
104 release of indigenous EPN, the release of exotic EPN species (but not exotic strains of
105 indigenous species) should be regulated due to greater potential risk. Thus, a discussion of the
106 risks posed by EPN must take into consideration the known geographical distribution and natural
107 habitats of the applied nematodes.

108 Surveys of EPN in Britain and Ireland have screened > 3000 soil samples collected from a
109 variety of habitats (e.g. grassland, woodland, heathland, hedgerows) (Blackshaw, 1988;
110 Hominick & Briscoe, 1990a & 1990b; Boag et al., 1992; Hominick et al., 1995; Gwynn &
111 Richardson, 1996; Chandler et al., 1997; Dillon, 2003). To date, there exist only two records of
112 *S. carpocapsae* in Britain (Georgis & Hague, 1979 & 1981), which have since been disputed (D.
113 Hunt, CABI Europe UK, pers. comm.), and no record of this species in Ireland. A recent, as yet
114 unpublished, study by Rae and colleagues has isolated *S. carpocapsae* from a gorse hedge and a
115 wooded layby, both in Cornwall. Both these isolates were far away from forestry with nematode
116 applications, but the authors are sequencing the mitochondrial DNA to be sure that they are
117 different from the BASF-Becker Underwood strains, which are used commercially (R. Rae,
118 LJMU UK, pers.comm.). While failure to detect a species does not confirm absence, based on
119 the available evidence we consider *S. carpocapsae* to be exotic to both Britain and Ireland (Table
120 1).

121 There are numerous records of *Steinernema feltiae* in Britain and Ireland (Blackshaw, 1988;
122 Griffin et al., 1991; Boag et al., 1992; Hominick et al., 1995; Gwynn & Richardson, 1996;

123 Chandler et al., 1997; Dillon, 2003), some of which are from coniferous forest soils (Hominick
124 & Briscoe, 1990a; Dillon, 2003; Harvey & Griffin, 2016). *Steinernema feltiae* strain 4CFMO
125 was isolated by Dillon (2003) from a coniferous clear-fell site in Ireland and we thus consider it
126 indigenous to this environment (Table 1). *Steinernema feltiae* strain EN02 is a commercially
127 produced strain (e-nema GmbH, Germany) that was originally isolated in Germany (Dillon et al.,
128 2008) and, though the species is indigenous to the UK and Ireland, we treat this strain as exotic
129 to Irish coniferous forest (Table 1). *Steinernema kraussei* has likewise been recorded in Britain
130 (Hominick et al., 1995), including in coniferous forest soil (Gwynn & Richardson, 1996). There
131 is one unpublished record of *S. kraussei* from a coniferous clear-fell site in Ireland, confirmed by
132 sequencing the rDNA internal transcribed spacer region (Harvey, unpublished data; Genbank
133 Accession numbers: KU847415, KU847416). Harvey collected *S. kraussei* from a Sitka Spruce
134 (*Picea sitchensis* [Bong.] Carr.) clear-fell from a soil sample around a stump after it had been
135 treated with *H. downesi* in Glendalough (53°03'N 006°28'W, elevation 300 m), which had been
136 felled in 2004. Samples were identified from two separate extractions from bulk samples of
137 several hundred to several thousand nematodes. There was some polymorphism detected, but this
138 is not unusual for the ITS region and has been observed before for *S. feltiae*. The Genbank blast
139 search confirmed the identity to be *S. kraussei* with 98-99% identity. *Heterorhabditis downesi* is
140 indigenous to Britain and Ireland, but has so far been isolated only from sandy coastal soils
141 (Griffin et al., 1994 & 1999). *Heterorhabditis megidis* has been isolated in Britain (Hominick et
142 al., 1995; Hominick, 2002), but has likewise not been reported in forest soils (Hominick &
143 Briscoe, 1990a; Gwynn & Richardson, 1996; Dillon, 2003). We therefore consider *H. downesi*
144 and *H. megidis* indigenous to Britain (and, in the case of *H. downesi*, also Ireland), but exotic to
145 coniferous forest plantations in the context of this case study (Table 1).

146 Risk categories for inundative control agents

147 Several methods to standardise risk assessment procedures for inundative control agents have
148 been proposed (van Lenteren et al., 2003; Babendreier et al., 2005; Mills et al., 2006). To meet
149 the criteria for risk assessment of introduced biological control agents recommended by the
150 Organisation for Economic Co-operation and Development (OECD, 2003), van Lenteren et al.
151 (2003) proposed a method of calculating a numerical index based on five risk categories. This
152 method allows for a categorical and quantifiable evaluation of risk. The index value is obtained
153 by estimating risk in each of the five categories based on specific criteria. The likelihood (very
154 unlikely to very likely) and magnitude (minimal to massive) of risk are each assigned a value of
155 1-5; the likelihood and magnitude values within each category are then multiplied and the
156 products are added to arrive at the final index value which can range from 5 to 125, where a
157 higher number indicates a greater environmental risk (van Lenteren et al., 2003). In the present
158 paper, we follow this approach, using results from the pine weevil system complemented by
159 literature from other contexts, to derive risk indices for EPN species *S. carpocapsae* (exotic to
160 Ireland), *S. feltiae* (one strain indigenous and one strain exotic to Ireland) and *H. downesi*
161 (indigenous to Ireland) when used against pine weevil in forestry. We have not included exact
162 risk values for *H. megidis* and *S. kraussei*, the other two species that have been tested against
163 pine weevil and for which fewer data are available, we estimate *H. megidis* to be similar to its
164 close relative *H. downesi*, both being exotic to the habitat, and *S. kraussei* to be similar to *S.*
165 *feltiae*, both species being present in the target habitat.

166

167

168 Risk of EPN application in forest ecosystem

169 a) Establishment

170 In inundative biological control, long-term persistence and establishment of the applied control
171 agent in the target environment is not a desired outcome (Bathon, 1996; van Lenteren et al.,
172 2003). Control agents are applied in large numbers to cause an immediate, but usually transient,
173 reduction in the pest population. EPN have the potential to persist in the soil after application
174 since the applied IJs are the non-feeding, stress-tolerant ‘dauer’ stage; in addition, they may
175 recycle and multiply in the field by infecting insects (Kaya & Gaugler, 1993; Grewal et al.,
176 2002). The extent and duration of post-application persistence of EPN is expected to vary with
177 the applied species, field conditions and the abundance and suitability of hosts (target and non-
178 target) (Smits, 1996; Barratt et al., 2010; Griffin, 2015). Though EPN numbers may be high in
179 the short term (weeks to months), in most studies numbers decrease rapidly over time and EPN
180 are usually no longer detectable within a year of application (Klein & Georgis, 1992; Wright et
181 al., 1993; Smits, 1996; Kurtz et al., 2007). In a minority of cases however, EPN have been
182 recorded more than a year after application (Shields et al., 1999; Susurluk & Ehlers, 2008;
183 Parkman et al., 1996).

184 Dillon et al. (2008a) investigated the persistence of EPN in soil around pine stumps treated to
185 suppress the large pine weevil in Irish trials. Four species were trialled: *H. megidis*, *H. downesi*,
186 *S. carpocapsae* and two strains of *S. feltiae*, a commercial strain (EN02) and an indigenous Irish
187 strain isolated from soil in a clear-felled coniferous forest (4CFMO) (Dillon, 2003; Dillon et al.,
188 2008a). EPN corresponding to the genus applied to a stump (i.e. *Steinernema* or *Heterorhabditis*)
189 were recovered up to three years after application (Dillon et al., 2008a), though recovery rates

190 decreased significantly over time: approximately 30 % of soil cores scored positive for EPN one
191 month after application, but only approximately 9 % did so after three years. Four and five years
192 after application, only *S. feltiae* was found, and it was recovered even around stumps treated with
193 other EPN species. When these *S. feltiae* isolates were compared to the applied strains
194 (indigenous 4CFMO and commercial EN02) using genome-wide molecular analysis (Amplified
195 Fragment Length Polymorphism, AFLP), they were found to be more closely related to the
196 indigenous strain 4CFMO than the exotic strain EN02 (Dillon et al., 2008a). Mesocosm
197 experiments with more controlled conditions by Dillon et al. (2008a) also showed greater
198 persistence of *S. feltiae* 4CFMO compared to *S. feltiae* EN02. Similarly, in a study conducted on
199 UK coniferous forest sites, Torr et al. (2007) compared the persistence of exotic *S. carpocapsae*
200 to that of indigenous *S. kraussei* (Table 1). One year after application, soil was sampled around
201 tree stumps treated with 3.5×10^6 IJs of either of the two species. There was a significant
202 decrease in levels of both species over time, though less rapidly for *S. kraussei* (Torr et al.,
203 2007). In addition, densities of *S. kraussei* were consistently higher than those of *S. carpocapsae*
204 from six months after application. Thus, both Torr et al. (2007) and Dillon et al. (2008a) found
205 that EPN species and strains exotic to the habitat persisted on clear-fell sites for shorter periods
206 than indigenous species or strains, possibly due to the latter being better adapted to the target
207 environment (Dillon et al., 2008a). We must, however, stress that detailed studies have been
208 undertaken only on a small sub-set of species and care must be taken when extending these
209 conclusions to other species given the variability in persistence reported among applied species.

210

211 Dillon et al.'s (2008a) study compared various species in a uniform setting (pine stumps on deep
212 peat soil), while Harvey and Griffin (2015) monitored persistence of a single species (*S.*

213 *carpocapsae*) under varied conditions: Lodgepole pine (*Pinus contorta* Douglas) and Sitka
214 spruce stumps on peat (nearly pure organic matter) or mineral soil. Similar to the results obtained
215 by Dillon et al. (2008a), the percentage of soil cores with *S. carpocapsae* decreased significantly
216 within the first two years after EPN application, from up to 12 % of cores after five months to 3
217 % after two years (Harvey & Griffin, 2016). Five years after application, only indigenous
218 *Steinernema* spp. were found around stumps (Harvey & Griffin, 2016). Similar results were
219 obtained for stump bark: *S. carpocapsae* was found under the bark of up to 67 % of stumps one
220 and two years after application, but was not detected there four or five years post application
221 (Harvey & Griffin, 2016). The incidence of *S. carpocapsae* was positively correlated with the
222 size of weevil populations in the stumps, suggesting that persistence of the EPN population was
223 dependent on the population of pine weevils, in which they can reproduce (Pye & Burman, 1978;
224 Dillon, 2003). Since stumps are suitable for pine weevil for only three to four years after felling
225 (Leather et al., 1999), and EPN are usually applied 12 to 18 months after felling (Dillon et al.,
226 2008a), this link between the target pest population and nematode persistence imposes a natural
227 limit on EPN recycling and, therefore, reduces the risk of long-term persistence and
228 establishment. A natural next step would be to extend these experiments to other EPN species,
229 which are potential inundative biological control agents for pine weevil.

230 We conclude that exotic *S. carpocapsae* and *H. downesi* as well as exotic strain *S. feltiae* EN02
231 used against the large pine weevil on clear-fell sites can persist by recycling in the target host in
232 the short term, but that establishment four years or more post-infection is ‘unlikely’ (likelihood =
233 2; Hickson et al., 2000; van Lenteren et al., 2003) (Table 2). Moreover, we consider the potential
234 non-target habitat on coniferous clear-fell sites where these exotic EPN may establish to be
235 ‘transient in time and space’ (van Lenteren et al., 2003), due to the apparent dependence of EPN

236 on pine weevils for recycling (magnitude = 1; van Lenteren et al., 2003; Table 2) though this has
237 only been experimentally determined for *S. carpocapsae*. This agrees with similar studies on
238 persistence in other, often very different settings (Smits, 1996; Susurluk & Ehlers, 2008). The
239 indigenous strain *S. feltiae* 4CFMO, however, was originally isolated from a coniferous clear-fell
240 site and so is likely to be adapted to this habitat and to hosts there, other than pine weevil.
241 Therefore, if it were applied to sites where it is not already present, it may persist for longer and
242 in a greater area compared to exotic EPN. We therefore conclude that establishment of *S. feltiae*
243 4CFMO on coniferous clear-fell sites is 'likely' (likelihood = 4; Hickson, 2000; van Lenteren et
244 al., 2003) and, because more than 50% of the area of coniferous clear-fell sites is soil available
245 for colonisation by EPN, the potential area of establishment is 'massive' (magnitude = 5; van
246 Lenteren et al., 2003) (Table 2). However, since it appears that native EPN may colonise clear-
247 fell sites as part of a natural ecological succession, following colonisation by native grasses and
248 the associated insect fauna (Harvey & Griffin, 2016), this 'risk' is essentially no different to that
249 of a natural recolonisation event. A less conservative view would be that the risk of
250 establishment for indigenous species necessarily represents the lowest risk possible and would
251 therefore better fit the category of 'very unlikely' establishment, resulting in a numerical risk
252 value of 1 for *S. feltiae* (van Lenteren et al., 20013). While establishment risk of EPN in
253 coniferous clear-fell soils can be considered low overall based on these results, persistence for up
254 to four years after application still provides a window of time in which they can disperse to other
255 areas, potentially creating additional risk.

256 b) Dispersal

257 EPN disperse through soil as IJs which are typically about 0.5 – 1 mm in length. Depending on
258 soil type, moisture content etc., the rate of horizontal dispersal of IJs after inundative application
259 is usually a few centimetres per day and limited to a scale of meters overall (Poinar & Hom,
260 1986; Downes & Griffin, 1996; Barratt et al., 2006). IJs of both *Steinernema* and *Heterorhabditis*
261 species can move through mineral and peat soils like those found on coniferous clear-fell sites
262 (Kruitbos et al., 2010; Williams et al., 2013). In addition, IJs may follow lateral roots
263 ('routeways') to locate and infect pine weevil larvae situated more than 50 cm from the point of
264 application (Dillon et al., 2006; Ennis et al., 2012).

265 Dillon et al. (2008a) investigated the dispersal of EPN in the field and in mesocosms containing
266 peat, simulating the type of soil typical of many coniferous plantations in Ireland and Britain. In
267 mesocosms, a very low incidence of three EPN species (*S. carpocapsae*, *S. feltiae* 4CFMO and
268 *H. downesi*) was detected 20 cm from the point of application, the maximum distance that was
269 sampled. In the field, soil samples were three to four times more likely to score positive for EPN
270 when taken at a treated tree stump compared to a distance of 20 cm from the stump (Dillon et al.,
271 2008a). The distance from the stump at which EPN were found was not influenced by species:
272 exotic species *S. carpocapsae* and *H. downesi* dispersed at a rate comparable to the indigenous *S.*
273 *feltiae* 4CFMO. Harvey & Griffin (2016) likewise observed that the probability of detecting *S.*
274 *carpocapsae* decreased significantly as distance from the stump increased from 0 cm to 60 cm.
275 These findings are in general agreement with previous studies in different settings, where EPN
276 presence decreases rapidly with distance from the point of application (Poinar & Hom, 1986;
277 Smits, 1996; Barratt et al., 2006; Jabbour & Barbercheck, 2008). However, care should be taken
278 when extrapolating these findings to other species not empirically tested.

279 Long-distance dispersal can occur, however, when facilitated by infected or externally
280 contaminated host insects or other carriers. Transport in wind and water may also occur, though
281 considered rare (Downes & Griffin, 1996; Griffin, 2015). The phoretic route is the most likely
282 explanation for reports of rapid short-range dispersal (Jabbour & Barbercheck, 2008) or long-
283 range dispersal over several hundred meters up to kilometres (Barratt et al., 2006). Following
284 application of *Steinernema scapterisci* (Nguyen and Smart, 1990) to control mole crickets in
285 Florida, infected insects were collected as far as 23 km from the nearest site of application
286 (Parkman et al., 1993 & 1996). Lacey et al. (1995) reported dispersal of *Steinernema glaseri*
287 (Steiner, 1929) IJs on the cuticle or within the haemocoel of *Popillia japonica* Newman, 1841.
288 Infected beetles in many cases contained enough nematodes to allow reproduction, and dispersal
289 in the field within infected hosts over at least 50 m was reported. The potential for dispersal of
290 EPN via attachment to and infection of adult pine weevils has been demonstrated in the
291 laboratory (Kruitbos et al., 2009).

292 Dillon et al. (2008a) tested for wider dispersal of EPN from treated stumps but found no EPN at
293 distances ranging from 1 to 10 m from the nearest treated stump. Harvey (2010) extended the
294 sampling up to 100 m off-site. *Steinernema carpocapsae* was detected in a small proportion of
295 samples collected 5 - 10 m from two of three sites where it had been applied 1-2 years previously
296 (Harvey, 2010). When the areas at which each of these positive samples was detected were
297 extensively re-sampled (40 bulk soil samples, each comprised of 5 subsamples at each previously
298 positive spot) five years after application, only native *Steinernema* spp. were isolated (Harvey &
299 Griffin, unpublished data). Failure to detect *S. carpocapsae* does not guarantee that no spread
300 and/or establishment of this species off-site has occurred, but it does suggest that any *S.*
301 *carpocapsae* populations that may have remained after five years are most likely small and

302 isolated. Similar tests for other EPN should be undertaken to establish their potential for off-site
303 spread.

304 The natural host range and the mechanisms underlying the persistence and patchy distribution of
305 EPN populations in the wild are poorly understood (Stuart & Gaugler, 1994; Peters, 1996; Smits,
306 1996; Griffin, 2015). However, given the results discussed here, the distance of dispersal within
307 and off clear-fell sites is unlikely to exceed 100 m (likelihood = 2; van Lenteren et al., 2003) for
308 any of the EPN investigated and, given the large number of IJs applied per stump (approx. $3.5 \times$
309 10^6), the magnitude of any such dispersal will probably be 'minimal' (i.e. $< 1\%$ of the applied
310 EPN dispersing, magnitude = 1; van Lenteren et al., 2003), which is similar to previous
311 evaluations of EPN dispersal risk (Smits, 1996; Barratt et al., 2006) (Table 2). The caveat here is
312 that these conclusions are based on detailed observations of a limited number of species; most
313 notably *S. carpocapsae* and that this risk factor may be revised in the light of future observations
314 on other EPN species applied inundatively in a forest ecosystem context.

315

316 c) Host range

317 In laboratory assays, EPN have a broad host range: for example, *S. carpocapsae* was reported to
318 kill >200 species of insects from 10 orders in close-contact laboratory assays (Poinar, 1979);
319 however, the realised host range in the field is expected to be much narrower, and the range of
320 insects affected to vary between species (Peters, 1996). Due to the wide potential host range,
321 however, van Lenteren et al. (2003) assigned maximal risk values of 5 to both likelihood and
322 magnitude of risk to *S. feltiae* when applied to an open field in Finland (> 30 species host range
323 and taxon range $>$ Order level, respectively; van Lenteren et al., 2003). We have adopted this

324 evaluation of host range for all EPN species used against the large pine weevil in our risk index
325 estimation (Table 2).

326

327 d) Direct non-target effects

328 Non-target impacts of inundatively applied EPN are of concern for three related reasons. Firstly,
329 negative impacts on biodiversity are considered detrimental in sustainable management of
330 natural resources, as they are likely to reduce the resilience and function of an ecosystem
331 (Bengtsson et al., 2000, Brockerhoff et al., 2008). Secondly, non-target insects that are of
332 particular benefit to sustainable forest management (e.g. wood decomposers) may be at particular
333 risk due to their proximity to the zone of nematode application (Harvey et al., 2012). Thirdly,
334 non-target impacts have the potential to disrupt natural control of the pest if they affect an
335 important natural enemy (van Lenteren, 2012; Harvey & Griffin, 2012). This last point is
336 underlined by the fact that control by natural enemies, without intervention, may make a
337 considerable economic contribution to pest control (Waage et al., 1988; Losey and Vaughan,
338 2006).

339 Direct non-target impacts arise when applied EPN infect and kill organisms other than the target
340 pest. Considering the wide potential host range of EPN (Peters, 1996), occasional infection of
341 non-target individuals is probably common when inundatively applying EPN IJs, but this should
342 be distinguished from widespread or pervasive non-target infection that reduces abundance and
343 diversity of non-target species (Bathon, 1996; van Lenteren et al., 2003). Published surveys of
344 non-target impacts at population and community level, before and after EPN application, suggest
345 that such impacts are rare and, if they do occur, tend to be minor (Bathon, 1996; Hodson et al.,

346 2002; Barratt et al., 2006). Nonetheless, plantation forests and the associated clear-fell sites,
347 though not always as diverse as mature and natural forest stands (Grove, 2002, Irwin et al.,
348 2014), may harbour a significant number of insects, particularly saproxylics, including red-listed
349 species (Sippola et al., 2002; Jonsell, 2007; Irwin et al., 2014). To assess the impact of EPN on
350 non-target insects in the pine weevil system we looked both for effects on community
351 composition and on two key ecosystem service providers, a parasitoid and a common saproxylic
352 species.

353 Saproxylic beetles, which develop in or feed on decomposing wood for at least part of their life
354 cycle, are considered beneficial in forest management and are, therefore, worth protecting
355 (Speight, 1989). These beneficial non-target insects may be at risk of infection as they occupy a
356 similar habitat to the pine weevil. The two-banded longhorn beetle *Rhagium bifasciatum*
357 Fabricius 1775 (Coleoptera: Cerambycidae) is an important wood-decomposing insect on clear-
358 fell sites in Europe (Duffy, 1953; Twinn & Harding, 1999). It develops over several years in
359 fallen deadwood and wood debris but, as tree stumps only become suitably decomposed for this
360 species three to four years after felling (Duffy, 1953), it usually does not co-occur with pine
361 weevils, which are present in stumps one to three years after felling (Leather et al., 1999). These
362 longhorns may, however, be impacted by misdirected spray during nematode application or by
363 EPN dispersing from treated stumps. Harvey et al. (2012) demonstrated that larvae, pupae and
364 adults of *R. bifasciatum* could be infected by both *S. carpocapsae* and *H. downesi* within
365 decomposing deadwood logs, though infection was significantly lower in field experiments than
366 in the laboratory. High rates of infection (> 30 % of insects) were typically only observed in logs
367 that had been directly drenched with a dose of 1.8 million IJs, half the number applied per stump
368 for pine weevil suppression (Dillon et al., 2008a). *Rhagium bifasciatum* infected with EPN were

369 also found in deadwood 1-12 months after application of *S. carpocapsae* to stumps on an
370 operational, site-wide scale, but fewer than 10% of logs contained infected insects, and infected
371 insects represented less than 4% of the overall population sampled. Both *S. carpocapsae* and *H.*
372 *downesi* reproduced in *R. bifasciatum* larvae, so it is possible that some of the infection was as a
373 result of recycling within the logs. The number of logs with infected *R. bifasciatum*, and number
374 of infected longhorns per log declined significantly with increasing distance of logs from treated
375 stumps (Harvey et al., 2012). The targeted application of EPN around tree stumps therefore
376 appears to limit direct non-target risks for this and probably also other saproxylic beetles in
377 deadwood and wood debris. However, tests of other EPN species, which may be used at an
378 operational level, would be required before we can be sure that this direct non-target effect is
379 minimal.

380 *Bracon hylobii* Ratzeburg 1848 is an important beneficial insect that provides natural control of
381 the large pine weevil (Henry & Day, 2001). Parasitism rates of pine weevil by this gregarious
382 ectoparasitoid are typically in the range of 15 – 30 % (Dillon et al., 2008; Harvey, unpublished
383 data), but can be as high as 90 % (Henry, 1995). Any intraguild predation of EPN on *B. hylobii*
384 could potentially be detrimental to this natural control (Rosenheim et al., 1995). Several
385 parasitoid wasps are susceptible to EPN, especially as larvae (Battisti, 1994; Lacey et al., 2003;
386 Mbata & Shapiro-Ilan, 2012). Larvae, pupae and adults of *B. hylobii* were susceptible to *H.*
387 *downesi* infection in laboratory assays (Everard et al., 2009). Adults emerging from cocoons
388 were most susceptible (80 % mortality in close-contact trials) while pupae inside cocoons were
389 infected only rarely (< 8 % of pupae infected inside cocoons after exposure to 10,000 IJs of *H.*
390 *downesi* [Everard et al., 2009]). However, such close-contact laboratory assays, with high
391 concentrations of EPN, almost certainly over-represent infection rates in the field. Dillon et al.

392 (2008b) found no reduction in *B. hylobii* parasitism of pine weevil in stumps treated with *H.*
393 *downesi* or *S. carpocapsae* 18 to 23 months earlier, but infection of *B. hylobii* itself with EPN
394 was not assessed. Susceptibility of a parasitoid to EPN does not necessarily impact on parasitism
395 of the pest: larvae of the parasitoid *Habrobracon hebetor* Say 1836 are susceptible to infection
396 with *Heterorhabditis indica* Poinar, Karunakar & David, 1992, but when nematode and wasp
397 were used together against Indian meal moth *Plodia interpunctella* Hübner 1813 in laboratory
398 assays, no antagonistic effect was observed (Mbata & Shapiro-Ilan, 2012). Tests of other EPN
399 species on *B. hylobii* would extend our confidence that there are minimal non-target effects.

400 Tree stumps can harbour a large diversity of invertebrates, both in the decomposing wood and
401 bark, and in the soil around them (Wallace, 1953; Abrahamsson & Lindbladh, 2006; Hedgren,
402 2007). Since this is where EPN are applied (Dillon et al., 2008a), impacts on non-target insects
403 are most likely to occur in this area. When debarking tree stumps to record infection of pine
404 weevil after application of EPN, infected non-target insects (e.g. Elateridae) were occasionally
405 found (Harvey, Dillon, pers. obs.). To monitor effects of EPN on non-target Coleoptera, Dillon et
406 al. (2012) placed insect emergence traps over stumps treated with *S. carpocapsae* or *H. downesi*
407 and over untreated stumps. EPN did not affect species diversity, richness, abundance or
408 community composition, either in the year of application or one year later (Dillon et al., 2012).
409 In particular, EPN application had no significant effect on wood-associated species including the
410 abundant saproxylic cerambycid, *Asemum striatum* L. 1758 (Dillon et al., 2012). The authors
411 concluded that the impact on non-target Coleoptera in and around tree stumps is probably
412 negligible for the two species tested to date.

413 Based on the available data summarized here, direct non-target impacts of the EPN species
414 investigated are ‘unlikely’ when applied against pine weevil (likelihood = 2; Hickson, 2000; van
415 Lenteren et al., 2003) (Table 2). In addition, data for both wood debris-associated and stump-
416 associated non-target insects suggest mortality of these insects is < 5 % of the total available
417 non-target population on site (magnitude = 1; van Lenteren et al., 2003). These assessments,
418 while supported by the limited data available for some EPN species, should be considered
419 tentative until further experimental data become available, especially for species whose non-
420 target risks have not yet been studied in detail in forest ecosystems.

421

422 e) Indirect non-target effects

423 Indirect effects of biological control are among the most difficult to study and disentangle
424 (Simberloff, 2012), making them the least researched aspect of risk assessment. Applying large
425 numbers of EPN may influence trophic interactions in the soil, thereby potentially changing
426 nematode (Somasekhar et al., 2002) and/or microarthropod assemblages (Hodson et al., 2002) as
427 well as nutrient cycles (De Nardo et al., 2006). Where persistence and dispersal of a control
428 agent are low risk factors, it can be argued that indirect non-target effects are also unlikely
429 (Barratt et al., 2006). Nonetheless, they should be assessed, for completeness. EPN may compete
430 for hosts with other parasites, pathogens and parasitoids at the same trophic level. In the pine
431 weevil system, we consider indirect effects on native EPN and on *Bracon hylobii*. Studies
432 elsewhere indicate that endemic nematodes may persist in spite of inundative application of EPN
433 (Miller and Barbercheck, 2001; Duncan et al., 2003). For example, Millar and Barbercheck
434 (2001) tested whether indigenous *S. carpocapsae* and *H. bacteriophora* were displaced by the

435 exotic nematode *Steinernema riobrave* (Cabanillas, Poinar, and Raulston, 1994) after inundative
436 application to corn fields in the US. Though the exotics persisted for more than two years, no
437 evidence of long-term displacement of either of the endemic species was found (Millar &
438 Barbercheck 2001). *Steinernema feltiae* was the only EPN recovered in a survey of coniferous
439 forestry throughout Ireland, being found in 10% of mature standing forests and 7% of replanted
440 clear-felled sites (Dillon, 2003), though *S. kraussei* has also been detected (Harvey,
441 unpublished). While *S. carpocapsae* was detected for at least 2 years following application, it
442 was replaced on several sites by indigenous steinernematids (Harvey and Griffin, 2016). As the
443 sites had not been sampled for EPN prior to treatment, it is not known whether endemic EPN
444 were temporarily suppressed to undetectable levels, or their later detection was as a result of a
445 new colonisation of the sites. Dillon et al. (2008a) found that the exotic species *S. carpocapsae*
446 and *H. downesi* and the exotic strain *S. feltiae* EN02 did not displace native strain *S. feltiae*
447 4CFMO on Irish clear-fell sites treated for pine weevil control. When applying an exotic strain of
448 an indigenous species, there is a risk of introgression (Roderick & Navajas, 2003; Hopper et al.,
449 2006), but there was no evidence of hybridization between indigenous and applied strains of *S.*
450 *feltiae* (Dillon et al., 2008a). These findings suggest that indigenous EPN species are unlikely to
451 be displaced in the long term by exotics that are not adapted to the target environment (Grewal et
452 al., 1994), but tests on further EPN species that may be used in pine weevil suppression activities
453 should be considered as the next step in the assessment of indirect non-target effects.

454 As previously noted, inundatively applied EPN may have direct effects on the parasitoid *B.*
455 *hylobii* by killing various life stages. We also consider the possibility of competition between
456 nematodes and this parasitoid for pine weevil larvae. *Bracon hylobii* cannot develop to adulthood
457 on hosts that have been infected with EPN; females oviposited on healthy host larvae, but not on

458 larvae killed by *H. downesi* or *S. carpocapsae*, which should reduce the negative impact on the
459 parasitoid (Everard et al., 2009; Harvey & Griffin, 2012). Female *B. hylobii*, especially those
460 with prior experience, did parasitize live hosts infected with EPN, as long as they were still
461 moving (Everard et al., 2009; Harvey & Griffin, 2012). While this means there is a possibility of
462 competition between EPN and *B. hylobii* (modulated by wasp experience), complementary
463 (additive or synergistic) control effects by the two agents may also emerge (Harvey & Griffin,
464 2012). Dillon et al. (2008b) reported an additive effect of *H. downesi* and *S. carpocapsae* with *B.*
465 *hylobii* on mortality of pine weevil in stumps across three sites. Larger-scale and longer-term
466 monitoring of *B. hylobii* populations is necessary to draw more definite conclusions about
467 population-scale effects of competition between EPN and *B. hylobii*.

468 We estimate that indirect non-target effects of exotic EPN species and strains used for large pine
469 weevil control (i.e. *S. carpocapsae*, *S. feltiae* EN02 and *H. downesi*) are ‘unlikely’ (likelihood =
470 2; Hickson, 2000; van Lenteren et al., 2003) (Table 2). and we expect these exotics to have only
471 a ‘minor’ impact on non-target organisms (magnitude = 2; van Lenteren et al., 2003) (Table 2).
472 Furthermore, we consider indirect non-target impacts to be ‘very unlikely’ for the native *S.*
473 *feltiae* 4CFMO (likelihood = 1; Hickson, 2000; van Lenteren et al., 2003) as it is already a
474 natural component of coniferous forest soils in Ireland and thus inundative application should not
475 have a qualitative impact on the soil organism community. It should be stressed, however, that
476 these assessments are based on the different aspects of indirect non-target impact investigated for
477 each of the species and that results for one species are not necessarily representative of others.
478 While we have not included exact risk values for *H. megidis* and *S. kraussei*, the other two
479 species that have been tested against pine weevil and for which fewer data are available, we

480 estimate *H. megidis* to be similar to its close relative *H. downesi*, both being exotic to the habitat,
481 and *S. kraussei* to be similar to *S. feltiae*, both species being present in the target habitat.

482

483 Conclusions and risk evaluation

484 Both exotic and indigenous EPN trialled against the large pine weevil persisted in the soil for up
485 to four years after application (Dillon et al., 2008a; Harvey & Griffin, 2016), but the evidence
486 suggests that persistence was driven by recycling through the target pest as intended.
487 Consequently, EPN levels decreased to background levels (for an indigenous strain) or
488 undetectable levels (for exotic species/strains) along with the natural decrease in pest population
489 (Torr et al., 2007; Dillon et al., 2008a; Harvey & Griffin, 2016). Moreover, the exotic applied
490 strain of *S. feltiae* did not displace an indigenous strain (Dillon et al., 2008a). Active horizontal
491 dispersal appeared to be limited to a zone of less than 1 m from the point of application and,
492 while phoresis or some other long-range mechanism of dispersal resulted in movement of EPN
493 outside the treated areas, there is no evidence that they established there (Dillon et al., 2008a;
494 Harvey & Griffin, 2016). Direct non-target effects are limited by the targeted application of
495 exotic EPN (Harvey et al., 2012) and coleopteran communities around tree stumps were
496 unaffected by exotic EPN (Dillon et al., 2012). Moreover, while the parasitoid *B. hylobii* is
497 susceptible to infection by and competition with EPN, there is no indication that this negatively
498 impacts on *B. hylobii* parasitism in the field (Dillon et al., 2008b; Everard et al., 2009; Harvey &
499 Griffin, 2012). Thus, both exotic and indigenous EPN seem to be well-suited as a low-risk
500 alternative to chemical pesticides.

501 Current risk considerations and regulatory restrictions on exotics have resulted in a trend to
502 favour indigenous inundative control agents over exotic ones, reversing the past emphasis on use
503 of exotics (van Lenteren, 2012). The results presented here do not suggest that risk, as defined by
504 van Lenteren et al. (2003), is increased by using exotic species. In fact, using EPN that are not
505 well-adapted to the environment where they are applied might reduce the risk of long-term
506 establishment (Grewal et al., 1994). The indexing method devised by van Lenteren et al. (2003),
507 when applied strictly, is only valid for the environment and setting in which the risk for the
508 control agent has been evaluated. In the setting of large pine weevil control using EPN, we
509 estimate the risk index of the exotic *H. downesi* and *S. carpocapsae* to be 35, as also for the
510 exotic strain of *S. feltiae*, EN02 (Table 2). We arrived at a somewhat higher index value of 51 for
511 *S. feltiae* 4CFMO (native) in a forestry setting in Ireland (Table 2). The main risk category
512 contributing to the differences in indices is establishment; we assign higher scores to the native
513 Irish species *S. feltiae*, particularly the native strain 4CFMO, as it has the potential to persist for
514 longer in coniferous clear-fell soils after application (Dillon et al. 2008a). However, since this
515 species already occurs naturally in this ecosystem, in this case a higher risk index value does not
516 necessarily imply a greater environmental hazard due to application. If we take the establishment
517 risk of *S. feltiae* to be the less conservative 1, then its index value becomes 36. By comparison,
518 van Lenteren et al. (2003) assign an index value of 53 to *S. feltiae* when released in Finland
519 (where it is indigenous) in an open field environment. The slightly different indices between the
520 two studies for application of a native *S. feltiae* are accounted for by higher estimates for
521 establishment and dispersal, and lower estimates for direct and indirect non-target effects in our
522 system compared to that of van Lenteren et al.

523 Of course, no risk assessment can ever be complete and offer a guarantee of safety – risks and
524 benefits must therefore always be weighed in sensible proportion to each other (Clerq et al.,
525 2011; Simberloff, 2012). The pine weevil has been controlled in Ireland and elsewhere mainly by
526 applying chemical pesticide (most recently cypermethrin or α -cypermethrin) to replanted
527 seedlings before and/or after planting (e.g. Torstensson et al., 1999; Willoughby et al., 2004).
528 EPN, as part of an integrated pest management strategy, are intended to help replace
529 cypermethrin and α -cypermethrin as their use is phased out in the European Union under
530 sustainable forest management (SFM) policies. An extensive body of research investigating
531 environmental impacts of pyrethroid pesticides in forestry shows that they can affect a much
532 wider range of organisms than do EPN (e.g. crustaceans and vertebrates), can impact on
533 terrestrial and – unlike EPN – also aquatic non-target organisms and can persist in both soil and
534 freshwater (e.g. McLeesc et al., 1980; Anderson, 1982; Kreutzweiser & Kingsbury, 1987;
535 DeLorenzo and Fulton, 2012). Moreover, by altering the composition of freshwater invertebrate
536 communities, pyrethroids can also have indirect impact on other non-target organisms
537 (Kingsbury & Kreutzweiser, 1987). Though the risk indexing method by van Lenteren et al.
538 (2003) is not designed to incorporate chemical pesticides, the risk of pyrethroids in terms of host
539 range, persistence (analogous to establishment for EPN) and direct and indirect non-target
540 impacts in the context of pine weevil control is likely to be greater than that of the EPN
541 discussed here. This is consistent with Laengle & Strasser (2010), who compared risk factors for
542 biological control agents with pesticides. They report risk factors in the order of thousands for
543 pesticides and in the order of hundreds for biological control agents. Thus, from the perspective
544 of minimizing the risk of environmental impact, EPN appear to be a superior alternative to
545 conventional chemical control methods when managing the large pine weevil.

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550 Pesticide Control Service of DAFM.

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553 Table 1: EPN species and strains for which risk assessment studies have been carried out in
 554 relation to pine weevil suppression. For each species and strain, status (exotic or indigenous) is
 555 given for Britain (Br) and Ireland (Irl) in general, and coniferous forest soils in these islands in
 556 particular. Risk categories after van Lenteren et al. (2003) are E = establishment, D = dispersal,
 557 DNT = direct non-target effects and INT = indirect non-target effects.

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EPN species	Strain and origin	Species/strain present in Br/Irl ¹	Species/strain present in coniferous forest soils? ¹	Risk categories Evaluated ¹
<i>Steinernema carpocapsae</i>	All strain, USA	Yes ² (1,2,3,5,7,8,11,12,22)	No ² (2, 8, 12, 13)	E, D, DNT, INT 15,16,18,19,20, 21
<i>Steinernema feltiae</i>	4CFMO, Ireland	Yes (1,4,5,7,8,11,12)	Yes (2, 12, 13)	E, D, INT 15
<i>Steinernema feltiae</i>	EN02, Germany	Yes ³ (1,4,5,7,8,11,12,15)	No ³ (15)	E, D, INT 15
<i>Steinernema kraussei</i>	Not specified (Torr et al. 2007)	Yes (7,8,11,13)	Yes (8,13)	E 14
<i>Heterorhabditis downsi</i>	K122, Ireland	Yes (6,11)	No (2,4,8,12)	E, D, DNT, INT 15,16,17,18,19, 20
<i>Heterorhabditis megidis</i>	UK211, UK; NL-HF85, Netherlands	Yes ⁴ (7,11)	No (2,4,8,12)	E, D, INT 15

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560 ¹References : [1] Blackshaw, 1988, [2] Hominick & Briscoe, 1990a; [3] Hominick & Briscoe, 1990b; [4]
 561 Griffin et al., 1991; [5] Boag et al., 1992; [6] Griffin et al., 1994; [7] Hominick et al., 1995; [8] Gwynn &
 562 Richardson, 1996; [9] Chandler et al., 1997; [10] Griffin et al., 1999; [11] Hominick, 2002; [12] Dillon,
 563 2003; [13] Harvey (unpublished data); [14] Torr et al., 1997; [15] Dillon et al., 2008a; [16] Dillon et al.,

564 2008b; [17] Everard et al., 2009; [18] Harvey et al., 2012; [19] Harvey & Griffin, 2012; [20] Dillon et al.,
565 2012; [21] Harvey & Griffin, 2016; [22] R. Rae, pers. comm. (2016)

566 ² *S. carpocapsae* has been found in Britain, but not Ireland.

567 ³ *S. feltiae* is present in UK and Ireland, but strain EN02 originated in Germany (Dillon et al., 2008a).

568 ⁴ *H. megidis* has been found in Britain, but not Ireland

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579 Table 2: Risk indices for *Steinernema carpocapsae*, *Heterorhabditis downesi* and *Steinernema*
580 *feltiae* when used against the large pine weevil. Values for likelihood of risk are determined on a
581 scale of 1 to 5 (1 = very unlikely, 2 = unlikely, 3 = possible, 4 = likely, 5 = very likely), as are
582 values for magnitude (1 = minimal, 2 = minor, 3 = moderate, 4 = major, 5 = massive), based on
583 criteria outlined in van Lenteren et al. (2003). Within each risk category, the values for
584 likelihood and magnitude of effects are multiplied, and the products are added to give the risk
585 index (van Lenteren et al. 2003).

EPN species/strain		Risk category					Risk index
		Establishment	Dispersal	Host range	Direct non-target effects	Indirect non-target effects	
<i>S. carpocapsae</i>	Likelihood	2	2	5	2	2	35
	Magnitude	1	1	5	1	2	
	L x M	2	2	25	2	4	
<i>H. downesi</i>	Likelihood	2	2	5	2	2	35
	Magnitude	1	1	5	1	2	
	L x M	2	2	25	2	4	
<i>S. feltiae</i> (EN02)	Likelihood	2	2	5	2	1	35
	Magnitude	2	1	5	1	2	
	L x M	4	2	25	2	2	
<i>S. feltiae</i> (4CFMO)	Likelihood	4	2	5	2	1	51
	Magnitude	5	1	5	1	2	
	L x M	20	2	25	2	2	
<i>S. feltiae</i> ¹	Likelihood	3	1	5	4	4	53
	Magnitude	5	1	5	2	1	
	L x M	15	1	25	8	4	

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587 ¹ The risk index for *S. feltiae* when applied to an open field in Finland from van Lenteren et al.
588 (2003) is given here for comparison.

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