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Harvey, CD, Williams, CD, Dillon, AB and Griffin, CT (2016) Inundative pest control: How risky is it? A case study using entomopathogenic nematodes in a forest ecosystem. Forest Ecology and Management, 380. pp. 242-251. ISSN 0378-1127

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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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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 or neonicotinoid insecticides.

31

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

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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 transport by mobile susceptible hosts (Downes & Griffin, 1996), they have the potential
50 to cause 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).

208

209 Dillon et al.'s (2008a) study compared various species in a uniform setting (pine stumps on deep
210 peat soil), while Harvey and Griffin (2015) monitored persistence of a single species (*S.*
211 *carpocapsae*) under varied conditions: lodgepole pine (*Pinus contorta* Douglas) and Sitka spruce
212 stumps on peat (nearly pure organic matter) or mineral soil. Similar to the results obtained by

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

228 We conclude that exotic *S. carpocapsae* and *H. downesi* as well as exotic strain *S. feltiae* EN02
229 used against the large pine weevil on clear-fell sites can persist by recycling in the target host in
230 the short term, but that establishment four years or more post-infection is ‘unlikely’ (likelihood =
231 2; Hickson et al., 2000; van Lenteren et al., 2003) (Table 2). Moreover, we consider the potential
232 non-target habitat on coniferous clear-fell sites where these exotic EPN may establish to be
233 ‘transient in time and space’ (van Lenteren et al., 2003), due to the apparent dependence of EPN
234 on pine weevils for recycling (magnitude = 1; van Lenteren et al., 2003; Table 2). This agrees
235 with similar studies on persistence in other, often very different settings (Smits, 1996; Susurluk

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

253 b) Dispersal

254 EPN disperse through soil as IJs which are typically about 0.5 – 1 mm in length. Depending on
255 soil type, moisture content etc., the rate of horizontal dispersal of IJs after inundative application
256 is usually a few centimetres per day and limited to a scale of meters overall (Poinar & Hom,
257 1986; Downes & Griffin, 1996; Barratt et al., 2006). IJs of both *Steinernema* and *Heterorhabditis*

258 species can move through mineral and peat soils like those found on coniferous clear-fell sites
259 (Kruitbos et al., 2010; Williams et al., 2013). In addition, IJs may follow lateral roots
260 ('routeways') to locate and infect pine weevil larvae situated more than 50 cm from the point of
261 application (Dillon et al., 2006; Ennis et al., 2012).

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

275 Long-distance dispersal can occur, however, when facilitated by infected or externally
276 contaminated host insects or other carriers. Transport in wind and water may also occur, though
277 considered rare (Downes & Griffin, 1996; Griffin, 2015). The phoretic route is the most likely
278 explanation for reports of rapid short-range dispersal (Jabbour & Barbercheck, 2008) or long-
279 range dispersal over several hundred meters up to kilometres (Barratt et al., 2006). Following

280 application of *Steinernema scapterisci* (Nguyen and Smart, 1990) to control mole crickets in
281 Florida, infected insects were collected as far as 23 km from the nearest site of application
282 (Parkman et al., 1993 & 1996). Lacey et al. (1995) reported dispersal of *Steinernema glaseri*
283 (Steiner, 1929) IJs on the cuticle or within the haemocoel of *Popillia japonica* Newman, 1841.
284 Infected beetles in many cases contained enough nematodes to allow reproduction, and dispersal
285 in the field over at least 50 m was reported. The potential for dispersal of EPN by adult pine
286 weevil has been demonstrated in the laboratory (Kruitbos et al., 2009).

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

299 The natural host range and the mechanisms underlying the persistence and patchy distribution of
300 EPN populations in the wild are poorly understood (Stuart & Gaugler, 1994; Peters, 1996; Smits,
301 1996; Griffin, 2015). However, given the results discussed here, the distance of dispersal within

302 and off clear-fell sites is unlikely to exceed 100 m (likelihood = 2; van Lenteren et al., 2003) for
303 any of the EPN investigated and, given the large number of IJs applied per stump (approx. 3.5 x
304 10⁶), the magnitude of any such dispersal will probably be ‘minimal’ (i.e. < 1 % of the applied
305 EPN dispersing, magnitude = 1; van Lenteren et al., 2003), which is similar to previous
306 evaluations of EPN dispersal risk (Smits, 1996; Barratt et al., 2006) (Table 2).

307

308

309 c) Host range

310 In laboratory assays, EPN have a broad host range: for example, *S. carpocapsae* was reported to
311 kill >200 species of insects from 10 orders in close-contact laboratory assays (Poinar, 1979);
312 however, the realised host range in the field is expected to be much narrower, and the range of
313 insects affected to vary between species (Peters, 1996). Due to the wide potential host range,
314 however, van Lenteren et al. (2003) assigned maximal risk values of 5 to both likelihood and
315 magnitude of risk to *S. feltiae* when applied to an open field in Finland (> 30 species host range
316 and taxon range > Order level, respectively; van Lenteren et al., 2003). We have adopted this
317 evaluation of host range for all EPN species used against the large pine weevil in our risk index
318 estimation (Table 2).

319

320 d) Direct non-target effects

321 Non-target impacts of inundatively applied EPN are of concern for three related reasons. Firstly,
322 negative impacts on biodiversity are considered detrimental in sustainable management of

323 natural resources, as they are likely to reduce the resilience and function of an ecosystem
324 (Bengtsson et al., 2000, Brockerhoff et al., 2008). Secondly, non-target insects that are of
325 particular benefit to sustainable forest management (e.g. wood decomposers) may be at particular
326 risk due to their proximity to the zone of nematode application (Harvey et al., 2012). Thirdly,
327 non-target impacts have the potential to disrupt natural control of the pest if they affect an
328 important natural enemy (van Lenteren, 2012; Harvey & Griffin, 2012). This last point is
329 underlined by the fact that control by natural enemies, without intervention, may make a
330 considerable economic contribution to pest control (Waage et al., 1988; Losey and Vaughan,
331 2006).

332 Direct non-target impacts arise when applied EPN infect and kill organisms other than the target
333 pest. Considering the wide potential host range of EPN (Peters, 1996), occasional infection of
334 non-target individuals is probably common when inundatively applying EPN IJs, but this should
335 be distinguished from widespread or pervasive non-target infection that reduces abundance and
336 diversity of non-target species (Bathon, 1996; van Lenteren et al., 2003). Published surveys of
337 non-target impacts at population and community level, before and after EPN application, suggest
338 that such impacts are rare and, if they do occur, tend to be minor (Bathon, 1996; Hodson et al.,
339 2002; Barratt et al., 2006). Nonetheless, plantation forests and the associated clear-fell sites,
340 though not always as diverse as mature and natural forest stands (Grove, 2002, Irwin et al.,
341 2014), may harbour a significant number of insects, particularly saproxylics, including red-listed
342 species (Sippola et al., 2002; Jonsell, 2007; Irwin et al., 2014). To assess the impact of EPN on
343 non-target insects in the pine weevil system we looked both for effects on community
344 composition and on two key ecosystem service providers, a parasitoid and a common saproxylic
345 species.

346 Saproxylic beetles, which develop in or feed on decomposing wood for at least part of their life
347 cycle, are considered beneficial in forest management and are, therefore, worth protecting
348 (Speight, 1989). These beneficial non-target insects may be at risk of infection as they occupy a
349 similar habitat to the pine weevil. The two-banded longhorn beetle *Rhagium bifasciatum*
350 Fabricius 1775 (Coleoptera: Cerambycidae) is an important wood-decomposing insect on clear-
351 fell sites in Europe (Duffy, 1953; Twinn & Harding, 1999). It develops over several years in
352 fallen deadwood and wood debris but, as tree stumps only become suitably decomposed for this
353 species three to four years after felling (Duffy, 1953), it usually does not co-occur with pine
354 weevils, which are present in stumps one to three years after felling (Leather et al., 1999). These
355 longhorns may, however, be impacted by misdirected spray during nematode application or by
356 EPN dispersing from treated stumps. Harvey et al. (2012) demonstrated that larvae, pupae and
357 adults of *R. bifasciatum* could be infected by both *S. carpocapsae* and *H. downesi* within
358 decomposing deadwood logs, though infection was significantly lower in field experiments than
359 in the laboratory. High rates of infection (> 30 % of insects) were typically only observed in logs
360 that had been directly drenched with a dose of 1.8 million IJs, half the number applied per stump
361 for pine weevil suppression (Dillon et al., 2008a). *Rhagium bifasciatum* infected with EPN were
362 also found in deadwood 1-12 months after application of *S. carpocapsae* to stumps on an
363 operational, site-wide scale, but fewer than 10% of logs contained infected insects, and infected
364 insects represented less than 4% of the overall population sampled. Both *S. carpocapsae* and *H.*
365 *downesi* reproduced in *R. bifasciatum* larvae, so it is possible that some of the infection was as a
366 result of recycling within the logs. The number of logs with infected *R. bifasciatum*, and number
367 of infected longhorns per log declined significantly with increasing distance of logs from treated
368 stumps (Harvey et al., 2012). The targeted application of EPN around tree stumps therefore

369 appears to limit direct non-target risks for this and probably also other saproxylic beetles in
370 deadwood and wood debris.

371 *Bracon hylobii* Ratzeburg 1848 is an important beneficial insect that provides natural control of
372 the large pine weevil (Henry & Day, 2001). Parasitism rates of pine weevil by this gregarious
373 ectoparasitoid are typically in the range of 15 – 30 % (Dillon et al., 2008; Harvey, unpublished
374 data), but can be as high as 90 % (Henry, 1995). Any intraguild predation of EPN on *B. hylobii*
375 could potentially be detrimental to this natural control (Rosenheim et al., 1995). Several
376 parasitoid wasps are susceptible to EPN, especially as larvae (Battisti, 1994; Lacey et al., 2003;
377 Mbata & Shapiro-Ilan, 2012). Larvae, pupae and adults of *B. hylobii* were susceptible to *H.*
378 *downesi* infection in laboratory assays (Everard et al., 2009). Adults emerging from cocoons
379 were most susceptible (80 % mortality in close-contact trials) while pupae inside cocoons were
380 infected only rarely (< 8 % of pupae infected inside cocoons after exposure to 10,000 IJs of *H.*
381 *downesi* [Everard et al., 2009]). However, such close-contact laboratory assays, with high
382 concentrations of EPN, almost certainly over-represent infection rates in the field. Dillon et al.
383 (2008b) found no reduction in *B. hylobii* parasitism of pine weevil in stumps treated with *H.*
384 *downesi* or *S. carpocapsae* 18 to 23 months earlier, but infection of *B. hylobii* itself with EPN
385 was not assessed. Susceptibility of a parasitoid to EPN does not necessarily impact on parasitism
386 of the pest: larvae of the parasitoid *Habrobracon hebetor* Say 1836 are susceptible to infection
387 with *Heterorhabditis indica* Poinar, Karunakar & David, 1992, but when nematode and wasp
388 were used together against Indian meal moth *Plodia interpunctella* Hübner 1813 in laboratory
389 assays, no antagonistic effect was observed (Mbata & Shapiro-Ilan, 2012)..

390 Tree stumps can harbour a large diversity of invertebrates, both in the decomposing wood and
391 bark, and in the soil around them (Wallace, 1953; Abrahamsson & Lindbladh, 2006; Hedgren,
392 2007). Since this is where EPN are applied (Dillon et al., 2008a), impacts on non-target insects
393 are most likely to occur in this area. When debarking tree stumps to record infection of pine
394 weevil after application of EPN, infected non-target insects (e.g. Elateridae) were occasionally
395 found (Harvey, Dillon, pers. obs.). To monitor effects of EPN on non-target Coleoptera, Dillon et
396 al. (2012) placed insect emergence traps over stumps treated with *S. carpocapsae* or *H. downesi*
397 and over untreated stumps. EPN did not affect species diversity, richness, abundance or
398 community composition, either in the year of application or one year later (Dillon et al., 2012).
399 In particular, EPN application had no significant effect on wood-associated species including the
400 abundant saproxylic cerambycid, *Asemum striatum* L. 1758 (Dillon et al., 2012). The authors
401 concluded that the impact on non-target Coleoptera in and around tree stumps is probably
402 negligible for the two species tested to date.

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

411

412 e) Indirect non-target effects

413 Indirect effects of biological control are among the most difficult to study and disentangle
414 (Simberloff, 2012), making them the least researched aspect of risk assessment. Applying large
415 numbers of EPN may influence trophic interactions in the soil, thereby potentially changing
416 nematode (Somasekhar et al., 2002) and/or microarthropod assemblages (Hodson et al., 2002) as
417 well as nutrient cycles (De Nardo et al., 2006). Where persistence and dispersal of a control
418 agent are low risk factors, it can be argued that indirect non-target effects are also unlikely
419 (Barratt et al., 2006). Nonetheless, they should be assessed, for completeness. EPN may compete
420 for hosts with other parasites, pathogens and parasitoids at the same trophic level. In the pine
421 weevil system, we consider indirect effects on native EPN and on *Bracon hylobii*. Studies
422 elsewhere indicate that endemic nematodes may persist in spite of inundative application of EPN
423 (Miller and Barbercheck, 2001; Duncan et al., 2003). For example, Millar and Barbercheck
424 (2001) tested whether indigenous *S. carpocapsae* and *H. bacteriophora* were displaced by the
425 exotic nematode *Steinernema riobrave* (Cabanillas, Poinar, and Raulston, 1994) after inundative
426 application to corn fields in the US. Though the exotics persisted for more than two years, no
427 evidence of long-term displacement of either of the endemic species was found (Millar &
428 Barbercheck 2001). *Steinernema feltiae* was the only EPN recovered in a survey of coniferous
429 forestry throughout Ireland, being found in 10% of mature standing forests and 7% of replanted
430 clear-felled sites (Dillon, 2003), though *S. kraussei* has also been detected (Harvey,
431 unpublished). While *S. carpocapsae* was detected for at least 2 years following application, it
432 was replaced on several sites by indigenous steinernematids (Harvey and Griffin, 2016). As the
433 sites had not been sampled for EPN prior to treatment, it is not known whether endemic EPN
434 were temporarily suppressed to undetectable levels, or their later detection was as a result of a

435 new colonisation of the sites. Dillon et al. (2008a) found that the exotic species *S. carpocapsae*
436 and *H. downesi* and the exotic strain *S. feltiae* EN02 did not displace native strain *S. feltiae*
437 4CFMO on Irish clear-fell sites treated for pine weevil control. When applying an exotic strain of
438 an indigenous species, there is a risk of introgression (Roderick & Navajas, 2003; Hopper et al.,
439 2006), but there was no evidence of hybridization between indigenous and applied strains of *S.*
440 *feltiae* (Dillon et al., 2008a). These findings suggest that indigenous EPN species are unlikely to
441 be displaced in the long term by exotics that are not adapted to the target environment (Grewal et
442 al., 1994), but tests on further EPN species that may be used in pine weevil suppression activities
443 should be considered as the next step in the assessment of indirect non-target effects.

444 As previously noted, inundatively applied EPN may have direct effects on the parasitoid *B.*
445 *hylobii* by killing various life stages. We also consider the possibility of competition between
446 nematodes and this parasitoid for pine weevil larvae. *Bracon hylobii* cannot develop to adulthood
447 on hosts that have been infected with EPN; females oviposited on healthy host larvae, but not on
448 larvae killed by *H. downesi* or *S. carpocapsae*, which should reduce the negative impact on the
449 parasitoid (Everard et al., 2009; Harvey & Griffin, 2012). Female *B. hylobii*, especially those
450 with prior experience, did parasitize live hosts infected with EPN, as long as they were still
451 moving (Everard et al., 2009; Harvey & Griffin, 2012). While this means there is a possibility of
452 competition between EPN and *B. hylobii* (modulated by wasp experience), complementary
453 (additive or synergistic) control effects by the two agents may also emerge (Harvey & Griffin,
454 2012). Dillon et al. (2008b) reported an additive effect of *H. downesi* and *S. carpocapsae* with *B.*
455 *hylobii* on mortality of pine weevil in stumps across three sites. Larger-scale and longer-term
456 monitoring of *B. hylobii* populations is necessary to draw more definite conclusions about
457 population-scale effects of competition between EPN and *B. hylobii*.

458 We estimate that indirect non-target effects of exotic EPN species and strains used for large pine
459 weevil control (i.e. *S. carpocapsae*, *S. feltiae* EN02 and *H. downesi*) are ‘unlikely’ (likelihood =
460 2; Hickson, 2000; van Lenteren et al., 2003) (Table 2), and we expect these exotics to have only
461 a ‘minor’ impact on non-target organisms (magnitude = 2; van Lenteren et al., 2003) (Table 2).
462 Furthermore, we consider indirect non-target impacts to be ‘very unlikely’ for the native *S.*
463 *feltiae* 4CFMO (likelihood = 1; Hickson, 2000; van Lenteren et al., 2003) as it is already a
464 natural component of coniferous forest soils in Ireland and thus inundative application should not
465 have a qualitative impact on the soil organism community. It should be stressed, however, that
466 these assessments are based on the different aspects of indirect non-target impact investigated for
467 each of the species and that results for one species are not necessarily representative of others.
468 While we have not included exact risk values for *H. megidis* and *S. kraussei*, the other two
469 species that have been tested against pine weevil and for which fewer data are available, we
470 estimate *H. megidis* to be similar to its close relative *H. downesi*, both being exotic to the habitat,
471 and *S. kraussei* to be similar to *S. feltiae*, both species being present in the target habitat.

472

473 Conclusions and risk evaluation

474 Both exotic and indigenous EPN trialled against the large pine weevil persisted in the soil for up
475 to four years after application (Dillon et al., 2008a; Harvey & Griffin, 2016), but the evidence
476 suggests that persistence was driven by recycling through the target pest as intended.
477 Consequently, EPN levels decreased to background levels (for an indigenous strain) or
478 undetectable levels (for exotic species/strains) along with the natural decrease in pest population
479 (Torr et al., 2007; Dillon et al., 2008a; Harvey & Griffin, 2016). Moreover, the exotic applied

480 strain of *S. feltiae* did not displace an indigenous strain (Dillon et al., 2008a). Active horizontal
481 dispersal appeared to be limited to a zone of less than 1 m from the point of application and,
482 while phoresis or some other long-range mechanism of dispersal resulted in movement of EPN
483 outside the treated areas, there is no evidence that they established there (Dillon et al., 2008a;
484 Harvey & Griffin, 2016). Direct non-target effects are limited by the targeted application of
485 exotic EPN (Harvey et al., 2012) and coleopteran communities around tree stumps were
486 unaffected by exotic EPN (Dillon et al., 2012). Moreover, while the parasitoid *B. hylobii* is
487 susceptible to infection by and competition with EPN, there is no indication that this negatively
488 impacts on *B. hylobii* parasitism in the field (Dillon et al., 2008b; Everard et al., 2009; Harvey &
489 Griffin, 2012). Thus, both exotic and indigenous EPN seem to be well-suited as a low-risk
490 alternative to chemical pesticides. While most of the risk assessment studies carried out in our
491 target forest ecosystem focussed on just two species, *S. carpocapsae* and *H. downesi*, we have
492 extrapolated our conclusions to *S. feltiae*. We feel this is acceptable, as *S. feltiae* as the species
493 indigenous to the system can be considered *a priori* of low risk.

494 Current risk considerations and regulatory restrictions on exotics have resulted in a trend to
495 favour indigenous inundative control agents over exotic ones, reversing the past emphasis on use
496 of exotics (van Lenteren, 2012). The results presented here do not suggest that risk, as defined by
497 van Lenteren et al. (2003), is increased by using exotic species. In fact, using EPN that are not
498 well-adapted to the environment where they are applied might reduce the risk of long-term
499 establishment (Grewal et al., 1994). The indexing method devised by van Lenteren et al. (2003),
500 when applied strictly, is only valid for the environment and setting in which the risk for the
501 control agent has been evaluated. In the setting of large pine weevil control using EPN, we
502 estimate the risk index of the exotic *H. downesi* and *S. carpocapsae* to be 35, as also for the

503 exotic strain of *S. feltiae*, EN02 (Table 2). We arrived at a somewhat higher index value of 51 for
504 *S. feltiae* 4CFMO (native) in a forestry setting in Ireland (Table 2). The main risk category
505 contributing to the differences in indices is establishment; we assign higher scores to the native
506 Irish species *S. feltiae*, particularly the native strain 4CFMO, as it has the potential to persist for
507 longer in coniferous clear-fell soils after application (Dillon et al. 2008a). However, since this
508 species already occurs naturally in this ecosystem, in this case a higher risk index value does not
509 necessarily imply a greater environmental hazard due to application. If we take the establishment
510 risk of *S. feltiae* to be the less conservative 1, then its index value becomes 36. By comparison,
511 van Lenteren et al. (2003) assign an index value of 53 to *S. feltiae* when released in Finland
512 (where it is indigenous) in an open field environment. The slightly different indices between the
513 two studies for application of a native *S. feltiae* are accounted for by higher estimates for
514 establishment and dispersal, and lower estimates for direct and indirect non-target effects in our
515 system compared to that of van Lenteren et al.

516 Of course, no risk assessment can ever be complete and offer a guarantee of safety – risks and
517 benefits must therefore always be weighed in sensible proportion to each other (Clerq et al.,
518 2011; Simberloff, 2012). The pine weevil has been controlled in Ireland and elsewhere mainly by
519 applying chemical pesticide (most recently cypermethrin or α -cypermethrin) to replanted
520 seedlings before and/or after planting (e.g. Torstensson et al., 1999; Willoughby et al., 2004).
521 EPN, as part of an integrated pest management strategy, are intended to help replace
522 cypermethrin and α -cypermethrin as their use is phased out in the European Union under
523 sustainable forest management (SFM) policies. An extensive body of research investigating
524 environmental impacts of pyrethroid pesticides in forestry shows that they can affect a much
525 wider range of organisms than do EPN (e.g. crustaceans and vertebrates), can impact on

526 terrestrial and – unlike EPN – also aquatic non-target organisms and can persist in both soil and
527 freshwater (e.g. McLeesc et al., 1980; Anderson, 1982; Kreutzweiser & Kingsbury, 1987;
528 DeLorenzo and Fulton, 2012). Moreover, by altering the composition of freshwater invertebrate
529 communities, pyrethroids can also have indirect impact on other non-target organisms
530 (Kingsbury & Kreutzweiser, 1987). Though the risk indexing method by van Lenteren et al.
531 (2003) is not designed to incorporate chemical pesticides, the risk of pyrethroids in terms of host
532 range, persistence (analogous to establishment for EPN) and direct and indirect non-target
533 impacts in the context of pine weevil control is likely to be greater than that of the EPN
534 discussed here. This is consistent with Laengle & Strasser (2010), who compared risk factors for
535 biological control agents with pesticides. They report risk factors in the order of thousands for
536 pesticides and in the order of hundreds for biological control agents. Thus, from the perspective
537 of minimizing the risk of environmental impact, EPN appear to be a superior alternative to
538 conventional chemical control methods when managing the large pine weevil.

539

540 **Acknowledgments:** CDH was funded by the EPA STRIVE programme (project 2007-PhD-B-6)
541 and CDW was funded by INTERREG IVA (IMPACT Project), co-funded by the Department of
542 Agriculture, Food and the Marine (DAFM). Field trials were conducted under licence from the
543 Pesticide Control Service of DAFM.

544

545 Table 1: EPN species and strains for which risk assessment studies have been carried out in
 546 relation to pine weevil suppression. For each species and strain, status (exotic or indigenous) is
 547 given for Britain (Br) and Ireland (Irl) in general, and coniferous forest soils in these islands in
 548 particular. Risk categories after van Lenteren et al. (2003) are E = establishment, D = dispersal,
 549 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	No (1,2,3,5,7,8,11,12)	N/A	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	Yes² (1,4,5,7,8,11,12,15)	No² (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 downesi</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	Yes³ (7,11)	No (2,4,8,12)	E, D, INT 15

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552 ¹References : [1] Blackshaw, 1988, [2] Hominick & Briscoe, 1990a; [3] Hominick & Briscoe, 1990b; [4]
 553 Griffin et al., 1991; [5] Boag et al., 1992; [6] Griffin et al., 1994; [7] Hominick et al., 1995; [8] Gwynn &
 554 Richardson, 1996; [9] Chandler et al., 1997; [10] Griffin et al., 1999; [11] Hominick, 2002; [12] Dillon,
 555 2003; [13] Harvey (unpublished data); [14] Torr et al., 1997; [15] Dillon et al., 2008a; [16] Dillon et al.,
 556 2008b; [17] Everard et al., 2009; [18] Harvey et al., 2012; [19] Harvey & Griffin, 2012; [20] Dillon et al.,
 557 2012; [21] Harvey & Griffin, 2016.

558 ² *S. feltiae* is present in UK and Ireland, but strain EN02 originated in Germany (Dillon et al., 2008a).

559 ³ *H. megidis* has been found in Britain, but not Ireland

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570 Table 2: Risk indices for *Steinernema carpocapsae*, *Heterorhabditis downesi* and *Steinernema*
571 *feltiae* when used against the large pine weevil. Values for likelihood of risk are determined on a
572 scale of 1 to 5 (1 = very unlikely, 2 = unlikely, 3 = possible, 4 = likely, 5 = very likely), as are
573 values for magnitude (1 = minimal, 2 = minor, 3 = moderate, 4 = major, 5 = massive), based on
574 criteria outlined in van Lenteren et al. (2003). Within each risk category, the values for
575 likelihood and magnitude of effects are multiplied, and the products are added to give the risk
576 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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578 ¹ The risk index for *S. feltiae* when applied to an open field in Finland from van Lenteren et al.
579 (2003) is given here for comparison.

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