Invasion by Fallopia japonica alters soil food webs through secondary metabolites

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9

10   Abstract

11

12   Biological invasions are a major threat to biodiversity with varying degrees of impact. There

13   is increasing evidence that allelopathy often plays an important role in explaining both

14   invasion success and impact on native taxa (e.g. novel weapons hypothesis). The effects of

15   these secondary metabolites on plant communities and microorganisms are well known.

16   However, their direct and indirect effects on soil fauna are unresolved, despite the

17   importance of the latter in ecosystem processes and, potentially, invasion mitigation.

18   Japanese knotweed (*Fallopia japonica*), an east-Asian species, which has proved to be

19   invasive in Europe, containing allelopathic secondary compounds inhibiting native plants

20   and microbial communities. The focal point of this study was the allelopathic effects of

21   knotweed on soil mesofauna (Nematoda, Collembola and Acari). During a one-month

22   laboratory experiment we added knotweed rhizome extract (KRE) at different

23   concentrations to soils collected in an invasion-prone area. He experiment consisted of

24 including or excluding secondary metabolites through the use of activated carbon filtration

25 of KRE. This enabled us to separate effects caused by nutrient addition (i.e. trophic effects)

26 and combined (trophic and allelopathic) effects. Relative effects of nutrient and secondary

27 metabolites addition on abiotic and biotic soil variables were then quantified. We

28 highlighted frequently contrasting trophic and allelopathic effects influenced in some cases

29 by KRE concentration. Microbial assemblages, through fungal / microbial biomass ratio, did

30 not show any congruent response to KRE secondary compounds but was more negatively

31 impacted by nutrient addition. The use of a trophic-based path analysis led us to show that

32 changes within the soil biota had repercussions on secondary consumers (e.g. bacterivorous

33 nematodes and Collembola). Abundance within taxa at higher trophic levels such as

34 predatory Acari (but not predatory nematodes) was also affected although to a lesser

35 extent, likely in part due to the limited considered timeframe. Overall, we showed that, in

36 controlled conditions, invasive allelopathic plants such as knotweeds can have effects on soil

37 fauna at different trophic levels through addition of both nutrients and secondary

38 metabolites to the soil. Considering the limited knowledge of allelopathic effects on the soil

39 fauna and soil functions, this study provides new information on above- and belowground

40 interactions.

41

45

46 1. Introduction

47

48 Past and current introduction of invasive plant species and their spread in new ecosystems

49 is a major concern for conservation at a global level (Litt et al., 2014; Pyšek et al., 2012) due

50 to their severe impact on biodiversity (Murrell et al., 2011; Vilà et al., 2011) and ecosystem

51 processes (Bassett et al., 2011; Kohyt and Skubała, 2013). Only a small number of exotic

52 species become invasive in their introduced range (Reinhart and Callaway, 2006) through

53 distinctive characteristics (or traits) providing superior competitive ability when compared

54 to native species (Van Kleunen et al., 2010). These traits can be morphological in nature by

55 directly improving plant fitness (Van Kleunen et al., 2010) or physiological with the synthesis

56 of biochemical, secondary metabolites that influence the germination, growth, survival

57 and/or reproduction of other organisms (Inderjit et al., 2011b).

58 The novel weapon hypothesis (NVH) suggests that the success of many exotic invasive plant

59 species is due to the possession of allelopathic compounds unencountered by native

60 species, particularly native plant species (Callaway and Ridenour, 2004). Furthermore, it has

61 been shown that many invasive species have different allelopathic potential effects between

62 their native and introduced ranges (Inderjit et al., 2011a; Thorpe et al., 2009). These

63 biochemical compounds, exudated from plant roots (Callaway et al., 2008) or released from

64 degrading litter (Inderjit et al., 2011a) have powerful effects on ecosystem functioning by

65 impacting both organisms and ecological processes (Hättenschwiler et al., 2011;

66 Hättenschwiler and Vitousek, 2000; Reigosa et al., 2006; Wardle et al., 1998).

67 Under the soil, plants interact with a wide range of organisms including bacteria, fungi,

68 nematodes and various kinds of arthropods (Abgrall et al., 2017; Parepa et al., 2013). These

69 aboveground-belowground relationships can be antagonistic (e.g. herbivores, pathogens) or

70 mutualistic (e.g. mycorrhizeous fungi, nitrogen-fixing bacteria) (Van der Putten et al., 2007).

71 Allelopathic biochemical that have a negative effect on plants can do so indirectly by

72 promoting or inhibiting particular soil biota (Callaway et al., 2008; Stinson et al., 2006).

73   Furthermore, the soil biota is known as having a structuring influence on plant community

74   composition, dynamics and phenology (Forey et al., 2015; Wardle, 2002), allelopathy

75   feedback from the soil biota could further increase invasion (Parepa et al., 2013).

76   Japanese knotweed (*Fallopia japonica* (Houtt.) Ronse Decr. 1988, Polygonaceae) was

77   introduced in Europe in the 19th century for its ornamental properties. It is now one of the

78   most destructive invasive species in Europe and North America (Lowe et al., 2000). *F.*

79   *japonica* spreads mostly by clonal rhizomatous growth with a single stem or rhizome node

80   being able to regenerate a full plant explaining the high-dispersion capacity of knotweed (De

81   Waal, 2001). Multiple species of the genus *Fallopia* such as *F. japonica, F. sachalinensis* as

82   well as their hybrid *F. ×bohemica* are known to contain and produce several secondary

83   metabolites (Murrell et al., 2011). Some of those compounds exhibit allelopathic properties

84   and can inhibit the germination or growth of other plant species (Aguilera et al., 2010;

85   Gerber et al., 2008) as well as bacteria (Hedenec et al., 2014) with mixed effects on fungi

86   (e.g. Daayf et al., 1995; Kumagai et al., 2005). A study by Vastano *et al.* (2000) revealed a

87   higher concentration of stilbenes in North American invasive *F. japonica* than in Chinese

88   native individuals of the same species tending to support the NVH in the case of knotweed.

89   One of these compounds, *trans*-resveratrol (3,4,5'-trihydroxystilbene), has been identified

90   as being produced by knotweed (Vastano et al., 2000). This molecule, which is also found in

91   grapevines, is known as having antifungal (Filip et al., 2003) and antibacterial properties

92   (Chan, 2002). Content analysis of resveratrol in knotweed tissues has been assessed by

93   Vaher & Koel (2003) who found that more than 80% of *trans*-resveratrol was located in the

94   roots and rhizomes, where the majority of plant-microorganisms interactions occur (Bais et

95   al., 2006).

96   Secondary metabolites present in knotweed rhizomes could have either a direct effect on

97   soil fauna either by repellence (Asplund et al., 2015), toxicity (Isman and Duffey, 1982) or an

98   indirect effect through changes in the soil biota (Ens et al., 2009). As evidence for direct

99   toxicity of phenolic compounds is scarce, indirect effects through alterations of basal

100   resources for secondary consumers appear more likely. In this paper, we studied the effect

101   of knotweed rhizome aqueous extracts on the soil biota and fauna in order to provide

102   additional information on the novel weapon hypothesis in this particular case. Indeed, while

103   several studies have assessed knotweed allelopathic potential in invaded areas none, as far

104   as we know, have considered the impact on the soil fauna in relation to this hypothesis.

105   Therefore and based on the theory, we hypothesized that: (1) knotweed has a negative

106   effect on microbial (and particularly bacterial) biomass through rhizome allelopathic

107   secondary metabolites ; (2) this negative effect has repercussions on higher trophic levels

108   through trophic cascades, and results in soil food web structure alteration ; (3) this negative

109   effect is be slightly attenuated by a positive trophic effect of nutrient addition provided by

110   knotweed rhizome extract ; (4) those effects, positive (i.e. trophic) or negative (i.e.

111   allelopathic), are concentration-dependent.

112

113   2. Material & Methods

114

115   2.1. Material collection and experiment preparation

116

117   Belowground *F. japonica* biomass was harvested in early autumn 2016 within a

118   spontaneously invaded plateau site in Normandy, France (49.455024° N; 1.062645° W). To

119   the best of our knowledge, control measures have never been applied to this site. Samples

120   were kept in an icebox for transportation to the laboratory. Rhizomes were water-cleaned

121   and stored at 4°C prior to extraction. We used an electric grinder to break plant tissues and

122   facilitate osmosis. One hundred grams of ground plant material was mixed with 1000 ml of

123   distilled water. This aqueous extract was kept at 19°C for 24h. Following Norsworthy (2003)

124   the mixture was then passed through a series of sieves ranging from 1000 to 50 µm and

125   then vacuum filtered through standard filter paper (> 20-25 µm). The extract was then

126   further sterilized by filtering through 0.22 µm filter.

127   We collected soil from the upper 10 cm of a reaped grassland in a small valley. While the

128   area was uninfested by *F. japonica* close-by sites (< 200 m) with similar topographical and

129   edaphic conditions have been invaded for several years. Macrofauna as well as macroscopic

130   plant materials were removed from the collected soil. Samples were gently, and

131   unforcefully, sieved at 5 mm so as to preserve mesofauna and mixed. Ten 200 g samples

132   were taken from the soil to sample initial Collembola, Acari and Nematoda communities.

133   Soils were placed in 8 x 8 x 10 cm plastic pots. Filter paper (<10-20 µm) was placed at the

134   bottom of the prevent leakage of the pot content. Sixty grams of fine grained sand was

135   added above the filter paper forming a ~ 0.5 cm layer. The rest of the pot was filled with 310

136   ± 5 g of soil. Ten 400 g samples of mixed soil were also collected for analysis of initial

137   physico-chemical conditions. A layer of 0.5 g of *Agropyron* sp. litter, which is the dominant

138   species in the samples grassland and also present close to invaded sites, was added to

139   provide physical habitat for the soil fauna. Pots were kept at 19°C in a phytotron with a 8h

140   day / 16h night cycle for a week. In order to increase and homogenize abundance and

141   compensate for possible losses during soil sieving each pot was then placed under 2

142   individual Berlese-Tullgren extractors, one containing topsoil (0 – 5 cm) and the other

143   deeper soil (5 – 10 cm) from the same area.

144

145   2.2. Experimental design

146

147   To simulate varying natural conditions and test for concentration-dependence distilled

148   water was used to provide different concentrations of the aforementioned aqueous KRE (0,

149   33, 66 & 100%). Half of the solution at each concentration, including distilled water, was

150   filtered 3 times through activated carbon prior to watering. This filtration was conducted in

151   order to remove potentially toxic organic compounds (Cheremisinoff and Ellerbusch, 1978)

152   from the KRE. We also filtered distilled water in order to test for the effect of filtration itself.

153   In total we obtained 8 different solution: filtered and unfiltered distilled water, filtered and

154   unfiltered 33% KRE, filtered and unfiltered 66% KRE as well as filtered and unfiltered 100%

155   KRE. Each solution was used to water 10 pots prepared as detailed above. The result was a

156   balanced factorial design (4 x 2 x 10 = 80 pots with 10 replicates per modality). During the

157   experiment, the pots were kept for four weeks (from early November to early December

158   2016) in a climate-controlled room (21.0 ± 1.9°C, 16h day / 8h night, 47.0 ± 8.8 % humidity)

159   and watered weekly with the corresponding solution.

160   2.3. Sampling, biochemical analysis and fauna identification

161

162   In order to verify the validity of our activated-carbon methodology we used HPLC to test for

163   resveratrol concentration in filtered and unfiltered KRE. Resveratrol ($C_{14}H_{12}O_3$), a phenolic

164   allelopathic compound, was measured using direct-injection high-performance liquid

165   chromatography (ThermoFisher Scientific UltiMate 3000 UHPLC). We used a variable

166   wavelength UV detector at 306 nm, equipped with a standard C18 column, a water-

167   acetonitril (60:40) mobile phase and an isocratic flow of 1 ml.min^{-1} (Goldberg et al., 1994).

168   We used commercially-available Resveratrol powder (CAS Number: 501-36-0) for calibration.

169   At the end of the experiment several biotic and abiotic variables were assessed.

170   Approximately 100g of fresh soil was used for springtail extraction in a Berlese-Tullgren

171   funnel (Macfadyen, 1961). Samples were weighted and placed within sieves (stitch: 1 mm,

172   diameter: 80 mm, height: 50 mm) above a plastic funnel. Extraction, under a heat source,

173   lasted for a week with individuals collected in 70% ethanol. This extraction method is

174   dependent on the limited tolerance of these animals to desiccation and will therefore only

175   extract active individuals. There is therefore no differentiation between individuals that

176   were inactivated, killed or otherwise incapacitated. One hundred grams of fresh soil was

177   used for nematode extraction in a Baermann funnel (McSorley and Walter, 1991).

178   Dampened samples were placed in a porous paper (10-15 μm stitch) supported by a 2 mm

179   sieve and placed above a water-filled and sealed funnel for 48h. This method has a limited

180   efficiency in isolating slow moving and nematodes and will not isolate inactive individuals

181   (Van Bezooijen, 2006) and thus is not exhaustive.

182   Mesofauna samples were separated into Acari and Collembola under a stereo binocular

183   microscope. Collembola individuals were mounted in lactic acid on microscope slides for

184   identification with a phase-contrast optical microscope. Collembola individuals were

185   identified to the species level (Hopkin, 2007; Potapov, 2001; Thibaud, 2004). Acari were

186   identified to the order or suborder level: Mesostigmata (Gamasida), Cryptostigmata

187   (Oribatida) and Prostigmata (Actinedida) (Coineau and Cleva, 1997). The cohort Astigmatina

188   (previously the suborder Astigmata) were included in the suborder Oribatida (Wang and

189   Fan, 2010). After Baermann funnel extraction, nematodes were counted while active under

190   a stereo binocular microscope. Following decantation nematodes were fixed using a 4%

191   formaldehyde solution and mounted on microscope slides. Individuals were attributed to

192   trophic groups (herbivores, bacterivores, fungivores and predators/omnivores) based on

193   mouthpart examination under a compound optical microscope.

194   Four grams of fresh soil were used to measure soil ergosterol content using the method

195   proposed by Gong, Guan & Witter (2001). Ergosterol is a sterol found within fungi and

196   protozoa. Ergosterol concentration can be used as a proxy of soil fungal biomass. Two times

197   20 g of fresh soil was used to assess microbial biomass using chloroform fumigation and

198   extraction (Brookes et al., 1985). Carbon extraction was performed in 100 ml of potassium

199   sulphate 0.05M (K_2SO_4) for chloroform-fumigated or unfumigated samples.

200   Once springtail extraction was complete a 30 g dry soil aliquot used to assess soil abiotic

201   variables. Soil pH was measured using 1:5 volumetric fraction in 1M potassium chloride (KCl)

202   using a Mettler Toledo FiveEasy pH meter. Total carbon and nitrogen content was measured

203   in a ThermoFisher Flash Analyzer 2000 after electric grinding of dry soil material.

204

205   2.4. Statistical analysis

206

207   We used ANOVA tests included in R Software 3.3.1 for statistical analysis. In order to assess

208   for knotweed rhizome extracts (KRE) effect on the soil fauna and microbiology we calculated

209   the relative differences between the considered modalities and our control by standardizing

210   and normalizing our measured values in relation to control means. This was done by

211   subtracting the average control value to each value for the considered modality. Based on

212   our methodology and hypotheses we considered that several of the potential effects of KRE

213   could be separated. We thus considered that subtracting the control mean to values

214   obtained in modalities where KRE was unfiltered by activated carbon (AC) values gave the

215   combined effect of KRE on the considered variable (Fig. 1). Based on the generally accepted

216   hypothesis we considered that AC filtration retains knotweed secondary compounds.

217   Therefore, subtracting control means to values measured in pots subjected to AC filtered

218   KRE we calculated KRE effect not attributable to secondary metabolites (Fig. 1). We referred

219   to this effect as a trophic effect that could directly affect soil microbiology by adding

220   nutrients to the substrate with potential indirect repercussions on higher trophic levels.

221   Finally, the same methodology was used by subtracting for each concentration the mean AC

222   filtered value to individual values measured in pots that received unfiltered KRE.

223   We used a null model approach in order to consider control stochastic variability. Null

224   vectors were randomly generated for each variable based on the observed distribution

225   parameters of the control. Significant differences between these null vectors and our data

226   vectors was assessed using non-parametric Wilcoxon signed-rank test. An iterative

227   procedure (one-thousand repetitions) and result aggregation enabled us to robustly, and

228   conservatively, assess for statistical differences between control and treatments. These

229   differences, when found, showed KRE effects on the considered variables.

230   We also assessed for differences in the structure of the soil food web with and without AC

231   filtration, and thus with or without knotweed secondary metabolites. We used multigroup

232   path analysis to model our empirically observed model and compare it to a "null"

233   multigroup model. The "null" model had constrained intercepts and regression coefficients

234   that was compared to the empiracl model using ANOVA. This approach provides a means to

235   assess for covariating responses of soil fauna compartments to potential allelopathy.

236

237   3. Results

238

239   3.1. Physico-chemistry & microbiology

240

241   Contrary to our hypothesis and the literature, we observed limited effect of knotweed

242   rhizome extracts (KRE) on microbiological variables. There were no significant differences in

243   ergosterol concentration, an indicator of fungal biomass, irrespective of concentration or

244   filtration mainly due to high variability. Regarding microbial carbon, a proxy of overall

245 microbial biomass, nutrient addition seems to cause a decrease as concentration increases

246 but insignificantly except at intermediate concentrations (-10.74 ± 4.04%; p < 0.05; Tab. 1)

247 with repercussions on ergosterol / microbial ratio (17.25 ± 6.06 ; p < 0.05; Tab. 1). pH was

248 also affected by KRE addition with a significant decrease in response to secondary

249 metabolites addition at the lowest concentration (-0.02 ± 0%; p < 0.05; Tab. 1) which shifted

250 to an increase at the highest concentration (+1.38 ± 0.5; p < 0.05; Tab. 1). The C/N ratio

251 remained largely unaffected by KRE input except at the highest concentration (combined

252 effect: -1.37 ± 0.56%; p < 0.05; Tab. 1).

253 Resveratrol concentration for 100% v/v KRE was 2.27 ± 0.23 mg.l^{-1} while the literature

254 suggests an IC_{50} (i.e. concentration for 50% mortality) of 9 mg.l-1 (Fan et al., 2010). We did

255 not detect resveratrol in any detectable amount in KRE samples after activated carbon

256 filtration, even at the highest concentrations. Several chromatograms detailing these

257 analysis are provided in Supplementary Material A1.

258

259 3.2. Nematodes

260

261 Total nematode abundance showed a strong response to KRE input at all concentrations

262 despite important differences in effect direction. For instance, total nematode abundance

263 was reduced by half following addition of low-concentration KRE (Fig. 2; Tab. 2). Conversely,

264 nematode abundance was almost doubled at the highest KRE concentration while no

265 response was found at the intermediate concentration (Fig. 2; Tab. 2). This general trend

266 (i.e. combined KRE effect) is the result of decreasing intensity and significance of responses

267 to nutrient addition (from +86 % at minimum concentration to +21 % at maximum

268 concentration; Fig. 2; Tab. 2) and highly contextual responses of nematodes to secondary

269   compounds addition (from -43 % at intermediate concentration to +48 % at maximum

270   concentration; Fig. 2; Tab. 2).

271   Although significance and intensity differed, bacterivorous and fungivorous nematodes

272   (41.07 ± 1.59 % and 22.35 ± 1.26 % of total nematodes abundance, respectively) response to

273   KRE addition varied similarly to the general trend with a shift from a negative (-43.5 % / -

274   79.8 %) to a positive (+58.3 % / +24.5 %) response with increasing KRE concentration (Tab.

275   2). Herbivorous nematodes abundance (28.0 ± 1.3 % of total abundance) varied somewhat

276   differently with a significant, and positive, response to KRE addition only at the highest

277   concentration (+177 % increase; Tab. 2). Nutrient addition appeared to elicit a generally

278   positive response independently of concentration (+64.8 ± 17.6 %) which was only

279   significant at the highest concentration (+59.2 ± 21.3 %; Tab. 2) while there was no

280   significant response to secondary compounds addition. Predatorous and omnivorous

281   nematodes showed no significant response to KRE addition (Tab. 2).

282

283   3.3. Mesofauna

284

285   Total Acari relative abundance showed a strong positive response to KRE input at all

286   concentrations without significant differences in intensity (from +138.1 to +223.8 % as

287   concentration increased; Fig. 3) which appears to me mostly related to a response to

288   secondary compounds addition. A similar pattern was observed for oribatid mites Tab. 3).

289   There were significant differences between responses at the lowest and highest

290   concentration levels for both the combined and secondary compounds responses but not

291   for response to nutrient addition (Tab.3). Predators (i.e. mostly Gamasida) abundances

292   responded only to the combined aspects of KRE addition, and only at the two lowest

293   concentrations (Tab. 3).

294   Regarding Collembola abundance observed response patterns are positive although only

295   significative when considering combined KRE effects at intermediate and high concentration

296   (+93.9 and +66.2 %, respectively; Fig. 4). A positive, yet unsignificant, effect of nutrient

297   addition seems to exist at the intermediate concentration (+56.9 ± 28.4 %; p < 0.10; Tab. 3).

298   Taxonomic diversity (i.e. Shannon's diversity) responded positively to KRE addition (only

299   significant at the two lowest concentrations). A response (positive) to secondary compounds

300   was only found at the intermediate KRE concentration (Tab. 3). Collembola functional

301   richness and evenness, calculated using trait data from the COLTRAIT database (Salmon et

302   al., 2014; Salmon and Ponge, 2012), only responded at 66% v/v KRE, mainly linked to the

303   secondary allelopathic effect of KRE addition. Functional evenness decreased while

304   functional richness increased in both cases (Tab. 3).

305

306   3.4. Path analysis

307

308   Differences between the empirically observed and a "null" multigroup model with

309   constrained intercepts and regression coefficients was tested using ANOVA. It showed a

310   difference in model structure between the two groups (i.e. unfiltered and activated carbon

311   filtered KRE addition) (n_{group1} = 40, n_{group2} = 40, Chi² difference = 39.87, p = 0.022).

312   Allelopathic effect removal through activated carbon (AC) filtration increased the strength

313   of the relationship between KRE concentration and microbial carbon concentration (From -

314   0.005 p = 0.816 to -0.032 p = 0.048; Fig. 5) and the effect of the latter on Collembola

315   abundance (-6.998 p = 0.384. to -14.443 p = 0.060; Fig. 5), herbo-fungivorous Acari

316   abundance (4.217 p = 0.530. to -9.884 p = 0.075; Fig. 5) and bacterivorous nematodes

317   abundance (0.864 p = 0.941. to -25.575 p = 0.027; Fig. 5). The relationship between

318   bacterivorous and predatorous nematodes abundance remained unaffected by AC filtration

319   (0.126 p = 0.000. to 0.111 p = 0.000; Fig. 5). The relationship between herbo-fungivorous

320   and predatorous Acari abundance was not significantly altered by AC filtration (0.030 p =

321   0.593. to 0.089 p = 0.176; Fig. 5).

322   Concerning the fungal pathway pathway allelopathic effect removal through activated

323   carbon (AC) filtration decreased the relationship, yet with still no significant relationship,

324   between KRE concentration and fungal biomass (i.e. ergosterol concentration) ($0.013^{\,p\,=\,0.174.}$

325   to $-0.007^{\,p\,=\,0.571}$; Fig. 5). Fungal biomass relationships with its consumers was also affected

326   by allelopathic effect removal: with herbo-fungivorous Acari ($18.681^{\,p\,=\,0.166.}$ to $-3.119^{\,p\,=\,0.693}$;

327   Fig. 5), Collembola abundance ($-1.464^{\,p\,=\,0.928.}$ to $17.470^{\,p\,=\,0.118}$; Fig. 5) and, in a very limited

328   way, fungivorous nematodes abundance ($-1.973^{\,p\,=\,0.836.}$ to $8.794^{\,p\,=\,0.274}$; Fig. 5). Strengths of

329   relationships of these taxa with their predators also changed after allelopathic effect

330   removal especially fungivorous nematodes ($-0.213^{\,p\,=\,0.011}$ to $-0.067^{\,p\,=\,0.348}$; Fig. 5) and

331   Collembola ($0.126^{\,p\,=\,0.009}$ to $-0.050^{\,p\,=\,0.282}$; Fig. 5) with predatorous Acari. The relationship

332   between fungivorous and predatorous nematodes abundance was mostly unaffected (-

333    $0.030^{\,p\,=\,0.593}$ to $0.089^{\,p\,=\,0.176}$; Fig. 5) as was the relationship between the two main

334   predatorous groups (i.e. predatorous nematodes and Acari) ($0.244^{\,p\,=\,0.161}$ to $-0.119^{\,p\,=\,0.496}$;

335   Fig. 5).

336

337   4. Discussion

338

339   The first hypothesis posited a negative effect of knotweed allelopathic secondary

340   compounds (ASC) on microbial communities as generally observed *in situ* (Hedenec et al.,

341   2014; Tamura and Tharayil, 2014). The effect of ASC addition was not directly tested on the

342   soil fauna, due in part to the lack of a proper identification of all such potential compounds

343   in knotweed (Fan et al., 2010). However, we were able to ascertain the removal of ASC from

344 a solution of knotweed rhizome extract (KRE) by using activated carbon which is known to

345 suppress allelopathic effects (Ridenour & Callaway, 2001 but see Lau et al., 2008 for a critic

346 of this methodology). We were able to test and demonstrate removal of one ASC, trans-

347 resveratrol, from KRE through activated carbon filtration (see Supplementary Material A1).

348 Differences in population responses between activated carbon filtered and unfiltered KRE

349 addition to the soil was therefore considered to be mainly, but not only, due to removal of

350 ASC. We then indirectly calculated a "secondary" effect of KRE. Contrary to our hypothesis,

351 we generally did not find any significant antimicrobial effects on ergosterol concentration (a

352 proxy of fungal biomass; Davis & Lamar, 1992), microbial carbon (a proxy of microbial

353 biomass; Vance et al., 1987) or the ratio between the two (a rough indicator of microbial

354 community structure; Djajakirana et al., 1996; Tab. 2). Ergosterol concentration itself

355 remained unaffected by KRE at concentrations which is consistent with the contrasting, yet

356 often positive, effects found in the literature on the effects on fungal biomass (Daayf et al.,

357 1995; Lecerf et al., 2007; Tamura and Tharayil, 2014). The pro-microbial, albeit insignificant,

358 effects are far more surprising and tend to refute our hypothesis and contrast with results

359 found in the literature (Hedenec et al., 2014; Kumagai et al., 2005; Stefanowicz et al., 2016;

360 Tamura and Tharayil, 2014). Most of these results were observed in the field, with multiple

361 potential confounding factors, with only Daayf et al., 1995 and Kumagai et al., 2005 directly

362 testing antimicrobial and antifungal properties of knotweeds secondary compounds in

363 controlled conditions. For instance, a major source of knotweed allelopathic properties are

364 linked to the slow degradation and release of phenolic compounds from leaf litter

365 degradation (Lavoie, 2017) which we did not account for in this study. Overall while the

366 antibacterial effect of knotweeds in general, and Japanese knotweed in particular, appear

367 fairly conclusive in the literature our results tend to show that this cannot be attributed to,

368 or only to, rhizome secondary metabolites.

369   The final hypothesis stipulated that knotweed KRE-addition effects, in particular ASC

370   addition, were linearly concentration-dependent. This hypothesis cannot be properly

371   segregated from the other and will be considered here to avoid repetition. This hypothesis

372   was based on the literature which frequently mentions release of secondary metabolites in

373   the environment by knotweeds as a major contributor to knotweed effects in their invasive

374   range (Vastano et al., 2000). Other lab studies have shown concentration-dependent effects

375   of some root-secreted phenolic compounds on microbial biomass (e.g. Zhang et al., 2015).

376   These compounds; however, are not in the same family as resveratrol or catechin. We

377   tested this hypothesis indirectly as the effect of secondary metabolites was calculated and

378   not measured. Concerning the concentration-dependence of microbiological response to

379   ASC the relationship remained unsignificant in all cases for both fungal and microbial

380   biomass (Tab. 1). Hence, while there may be concentration-dependence the effects

381   themselves are unsignificant, and we must therefore accept the alternative hypothesis in

382   the case of ASC. Nutrient addition, however, significantly and negatively affected microbial

383   biomass at 66% v/v concentration only, showing differences dependent on concentration.

384   However, as this is for intermediate concentration there does not appear to be a linear

385   relationship between concentration and response. When analyzing changes in soil food web

386   structure we showed that allelopathic secondary compounds (ASC) removal increased the

387   strength of the negative correlation between KRE concentration and microbial biomass, the

388   corollary being that ASC addition would tend to decrease the strength of the relationship

389   between the two (Fig. 5). Only after ASC removal was the relationship significant between

390   the two variables. Therefore, while the combined, and ASC, effects on microbial biomass

391   appear not to be concentration-dependent purely "trophic" effects are significantly so.

392   The second hypothesis stated that, if there were antimicrobial and antifungal effects of KRE,

393   they would have repercussions on higher trophic levels through a trophic cascade with

394   potential alterations of trophic structures. We showed previously that no antimicrobial or

395   antifungal effect were found in our experiment in response to KRE of allelopathic secondary

396   compounds (ASC). However, the evidence revealed significant differences in abundance at

397   higher trophic levels. Indeed, we did find significant effects of KRE on bacterivorous and

398   fungivorous nematodes (both positive and negative) abundances as well as herbo-

399   fungivorous Acari and Collembola abundances (mostly positive) from both allelopathic or

400   combined effects (although with no consistent pattern at different concentrations; see Tab

401   2-3). We found no significant differences in predator nematodes abundances despite the

402   changes in abundance of their prey. Predator Acari, on the other hand, had higher

403   abundances following KRE addition. Accordingly, predators may have had a top-down effect

404   on microbivores while being unaffected themselves by bottom-up regulation themselves in

405   the considered timeframe. Unfortunately, we could not find any reports in the literature on

406   nematodes abundances under knotweed and therefore cannot assess the representativity

407   of our results. Skubala & Mierny (2009), on the other hand, found a significant negative

408   effect of giant knotweed (*Fallopia sachalinensis*) on oribatid mites (mostly herbivores,

409   fungivores or both) but no effect on Collembola abundance (mostly generalist fungivores

410   and detrivores). They attributed observed effects in the field on liberation phenolic

411   compounds from leaf litter degradation, not rhizome excretion. This is, to our knowledge,

412   the only publication to date assessing knotweed effects on the soil mesofauna in

413   spontaneously invaded sites. Hedenec et al. (2014) also considered Collembola and Acari,

414   with mixed results and no differences with native species, but in an agricultural setting with

415   giant knotweed used as a biofuel crop. Macroarthropod abundance, which we did not

416   consider in this study, has generally been shown to be negatively affected by knotweed

417   presence (Gerber et al., 2008; Kappes et al., 2007; Topp et al., 2008)

418   Collembola and Acari have a generation time of several weeks to months, depending on

419   taxa and eco-morphological group (Choi et al., 2002; Ermilov and Lochynska, 2008; Joosse

420   and Veltkamp, 1969; Park, 2007; Prinzing et al., 2002; Verhoef and Selm, 1983). Observed

421   differences are thus unlikely to be caused by a predator-prey intergenerational regulation

422   but would be due to a more direct effect. Predatory nematodes and Acari have generation

423   times ranging from 3 to 280 days depending on taxa (Abou-Awad et al., 2001; Khan et al.,

424   2007; Ydergaard et al., 1997) as well as temperature. For some taxa within our study the

425   experiment duration, 1 month, may have been insufficient to observe significant

426   repercussions on higher trophic levels through intergenerational predator-prey

427   relationships.

428   We also assessed how activated carbon filtration, and thus ASC removal, affected the

429   structure of the soil trophic network by using multigroup path analysis (Fig. 5). The

430   comparison of an empirical model to a constrained model, clearly showed that ASC removal

431   significantly altered overall relationships between the various considered faunal groups. In

432   this analysis, ASC removal also seemed to more readily alter relationships between taxa in

433   the microbial food web (i.e. between microbial biomass and abundances of Collembola,

434   oribatid mites and bacterivorous nematodes). This relationship was, however, negative. This

435   would suggest that ASC removal from added KRE increases the interdependency of

436   compartments/taxa within the microbial food web as well as the effect of KRE concentration

437   on microbial biomass. The corollary, although we did not test it directly, would be that the

438   allelopathic component of knotweed effect tends to limit between-group variability. In

439   addition, it would appear that while nutrient input has concentration-dependent effects (at

440   least on microbial biomass), nutrient and allelopathic effects are not dependent on

441   concentration. Another striking feature is the change in the relationship between

442   fungivorous nematodes and predatorous Acari, for which intensity was drastically reduced

443   by ASC removal from KRE and most importantly switched from a negative to a positive

444   correlation.

445   We also posited that part of knotweeds success as invaders was due to lack of adaptation by

446   native species to the invader: the novel weapon hypothesis (Callaway and Ridenour, 2004).

447   This framework most generally applies to other plant species with which there is more

448   direct competition. While our methodology does not enable us to assess that effect, we

449   expected negative effects (on abundance and diversity) of knotweed allelopathy on at least

450   some taxa within the soil fauna which would help explain results found in several field

451   studies (Gerber et al., 2008; Kappes et al., 2007; Skubala and Mierny, 2009; Topp et al.,

452   2008). Such negative effects could have been direct through phytotoxicity or indirect

453   through a trophic cascade or changes in habitat structure. This was not the case here with

454   few negative responses of soil fauna to ASC addition. The only significant negative effects

455   documented were for all types of nematodes in response to 66% KRE addition (Tab. 2).

456   The third hypothesis centered on the posited countering of negative allelopathic effects of

457   KRE addition by nutrient addition. This appears to be the case, albeit unsignificantly, for the

458   ergosterol / microbial C ratio at the two highest concentrations which is negatively affected

459   by the allelopathic component of KRE (i.e. favoring microbial biomass) and positively

460   affected by the nutrient-addition component (i.e. favoring fungal biomass) with a combined

461   positive effect. Field studies evaluating fungal:bacterial ratios have found conflicting results

462   on that matter with both increased (Suseela et al., 2016; Tamura and Tharayil, 2014) and

463   decreased (Stefanowicz et al., 2016) ratios under knotweed-invaded plots. The results tend

464   to support the first case of decreased bacterial biomass, although fungal biomass remained

465   unaffected (Tab. 1). These effects of knotweed are often attributed to increased litter

466   biomass (e.g. Suseela et al., 2016) and changes in litter chemistry (higher litter C/N ratios:

467   Dassonville, Guillaumaud, Piola, Meerts, & Poly, 2011; Mincheva et al., 2014; Urgenson,

468   Reichard, & Halpern, 2009; higher litter lignin content: Aguilera et al., 2010) in addition to

469   the already mentioned allelopathic effects. Our decomposition of effects between nutrient

470   and secondary compounds addition tends to indicate that this positive effect on

471 fungal:microbial ratios is mainly attributed to a positive response of microbial biomass to

472 increased nutrient input to the soil. Due to lack of leaf litter this is not comparable to field

473 nutrient input, but nonetheless worth considering. Secondary compounds, which can be

474 twice as concentrated in knotweed-invaded plots (Suseela et al., 2016), appear to have a

475 negative effect on fungi:microbial biomass ratios in this case. Changes in microbial biomass

476 carbon have the most influence over shits in this ratio in our case (i.e. negative effect of

477 nutrient-addition and positive effect of secondary compounds addition) which would to be

478 contrary to our hypothesis. The pH responded in a similar manner with a significant negative

479 nutrient addition effect, a significant positive secondary compounds effect but an

480 unsignificant slightly positive combined effect. A decrease in soil pH has generally been

481 observed in field studies (Dassonville et al., 2011, 2008; Kappes et al., 2007) and is also

482 attributed to increased litter biomass in knotweed stands. This is, however, not always the

483 case (Stefanowicz et al., 2017; Stoll et al., 2012)

484 Finally the results regarding microarthropod abundances (i.e. Collembola and Acari) are

485 ambiguous with no evidence of the hypothesized pattern of attenuating effects. In fact, all

486 significative responses of both Acari and Collembola are positive. This would seem, contrary

487 to our hypothesis, to indicate a synergetic combined effect of KRE addition (mostly at

488 intermediate and high concentrations). In all cases of significant response to a combined

489 effect one or both components' response was neutral (nutrient addition especially).

490 Contrasting and inverse results from nutrient and secondary metabolites addition have also

491 been found for nematodes at intermediate KRE concentration with a negative ASC addition

492 effect, positive nutrient addition effect and neutral combined effect. Direct nematocidal

493 effects of plant secondary compounds have been documented in some cases, mostly in

494 laboratory studies (Chitwood, 2002). If there was such a direct effect of knotweed we would

495 expect it to also be present at the highest concentration, which is not the case in our study

496 (Fig. 2).

497   These results provide the basis for further research on knotweed such as more detailed

498   characterization of knotweed ASC and their potential allelopathic effects as well as further

499   field work. As assessment of indirect, and direct, allelopathic effects of phenolic compounds

500   on soil fauna has rarely been done. Thus, this report should provide useful data for authors

501   working on such a subject as information is currently scarce on the subject. Finally, we hope

502   the results presented here will provide useful reference data for future biological invasions

503   study and inform managers of invaded areas on knotweed potential impacts.

504

505   5. Conclusion

506

507   In conclusion, the results showed an effect of knotweed rhizome extract (KRE) on soil

508   microbiology. Fungal biomass remained unaffected but microbial biomass as a whole

509   responded negatively to KRE in some cases. Interestingly these negative responses, when

510   they occurred, were mostly attributable to factors other than the allelopathic secondary

511   compounds (ASC) within KRE, most notably nutrient addition. Calculated responses of

512   microbial biomass to ASC addition were, albeit unsignificantly so, positive. While KRE

513   addition had an effect in most cases on taxa "higher" within the soil trophic networks, there

514   were no evident and generalizable trophic cascades across trophic levels for a given KRE

515   concentration. Path analysis did reveal important changes in soil food web structure

516   (constructed based on hypothesized producer-consumer relationships) which appeared to

517   be mostly within the bacterial pathway, and concentration-dependent. There was

518   circumstantial, but not generalizable, evidence of compensating, or attenuating, effects of

519   nutrient and ASC addition on various taxa. Rarely was ASC effect, when documented,

520   concentration-dependent in the results.

521

532   7. References

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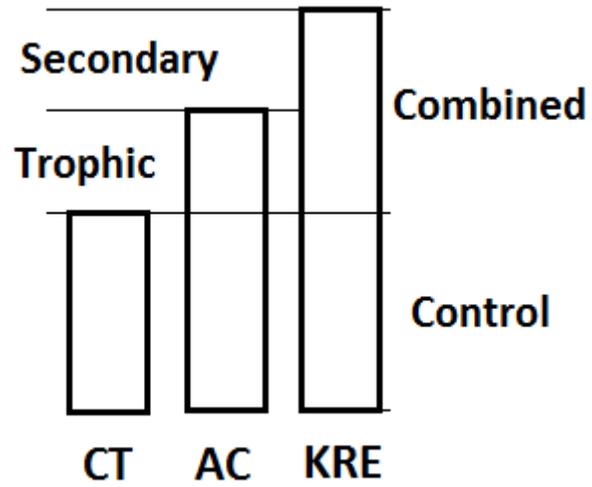742   belowground components. Princeton University Press.

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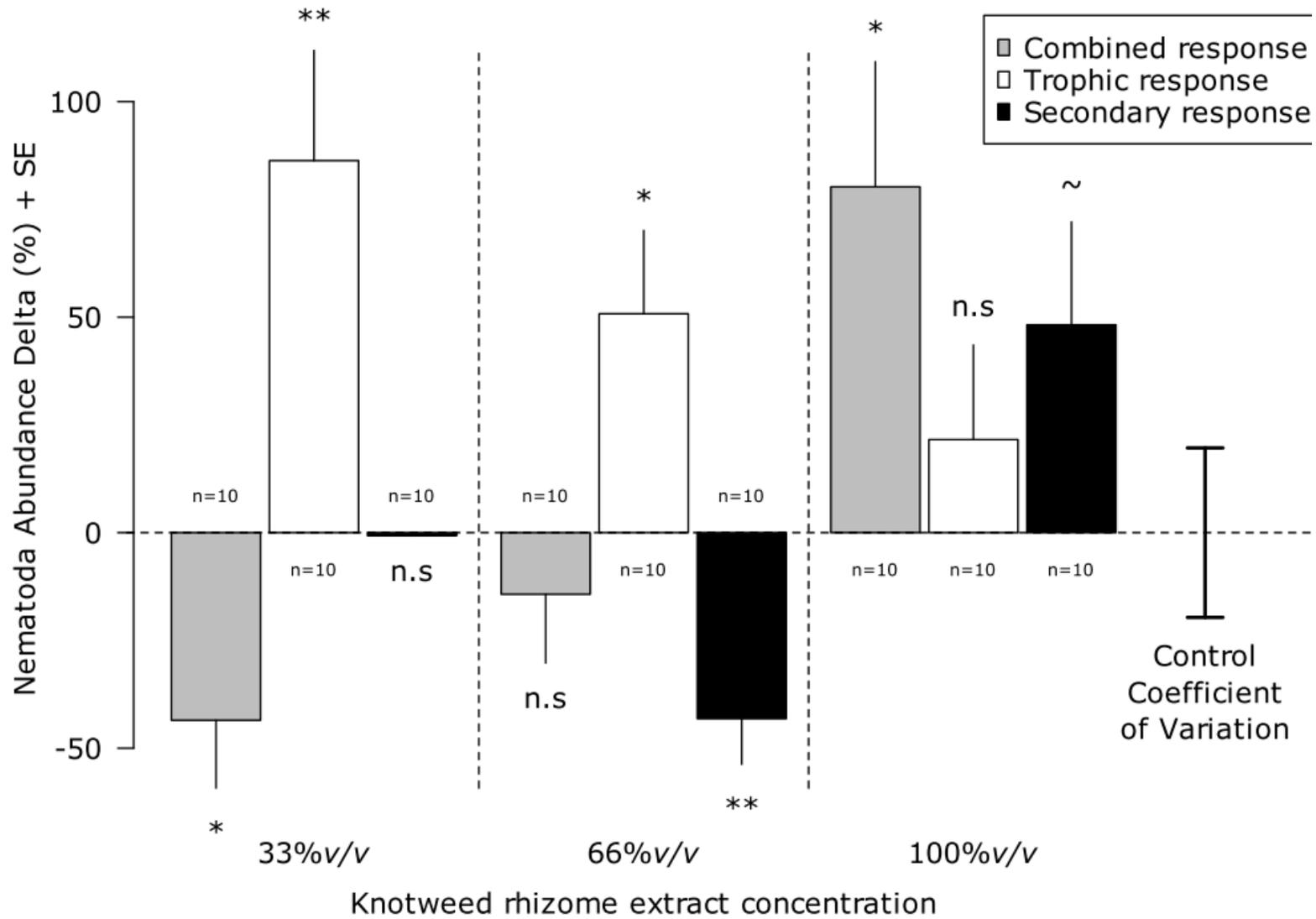
751

753   Figures

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755   Figure 1 : Diagram of calculations involved in separating knotweed rhizome extract (KRE) effects. CT: control, AC: activated carbon filtration, KRE:

756   no AC filtration.

757

758   Figure 2 : Relative nematode total abundance (%) compared to control in relation to knotweed rhizome extract dilution levels and activated carbon

759   filtration with decomposition of effects. Symbols indicate levels of significativity of repeated statistical testing of differences between calculated

760   values and null generated controls. n.s.: $p > 0.10$, ~ : $p < 0.10$, * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$, **** : $p < 0.0001$.

761

762   Figure 3 : Relative Acari total abundance compared to control in relation to knotweed rhizome extract dilution levels and activated carbon filtration

763   with decomposition of effects. Symbols indicate levels of significativity of repeated statistical testing of differences between calculated values and

764   null generated controls. n.s.: $p > 0.10$, ~ : $p < 0.10$, * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$, **** : $p < 0.0001$.
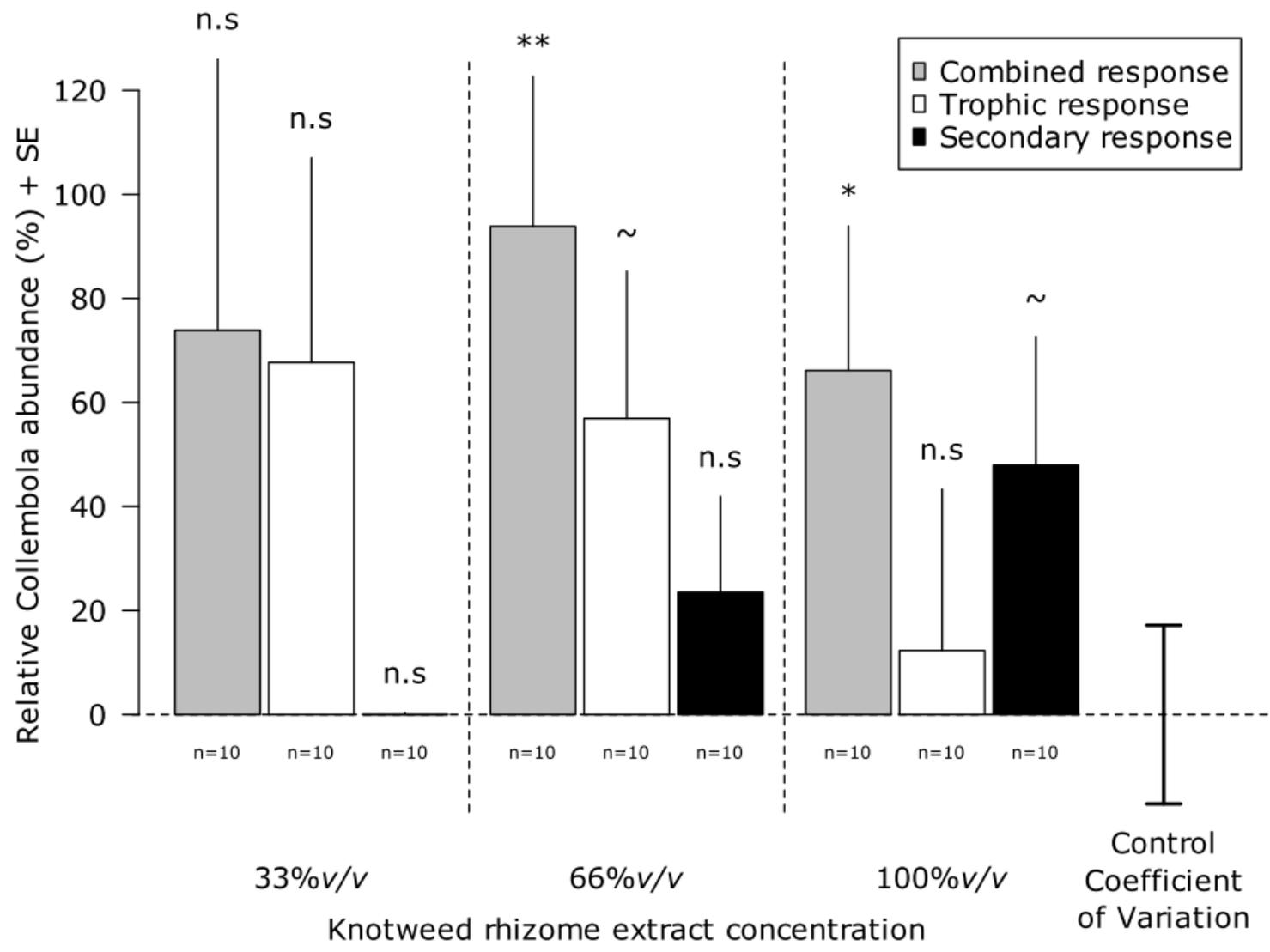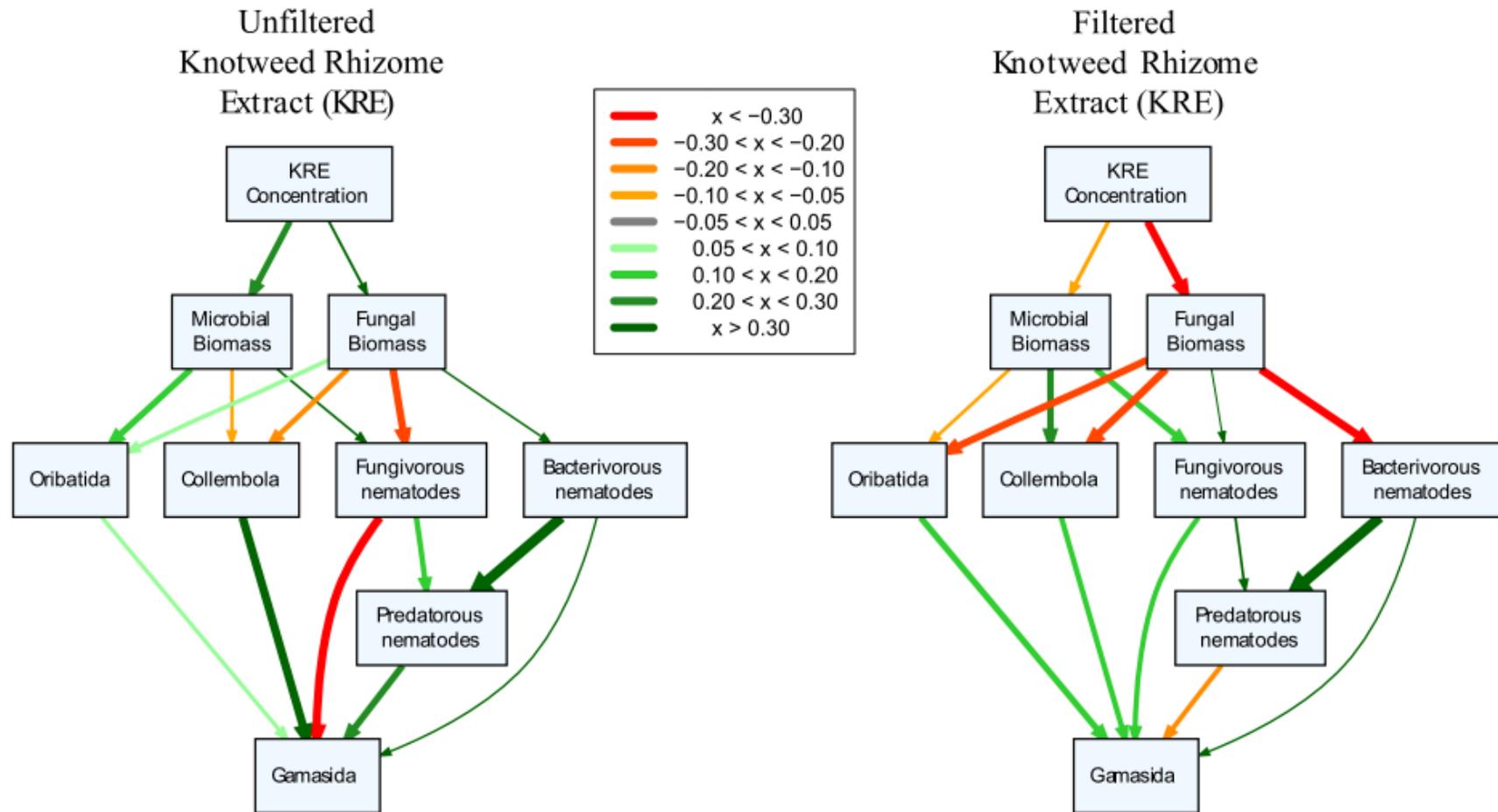
765

766   Figure 4 : Relative Collembola total abundance compared to control in relation to knotweed rhizome extract dilution levels and activated carbon

767   filtration with decomposition of effects. Symbols indicate levels of significativity of repeated statistical testing of differences between calculated

768   values and null generated controls. n.s.: $p > 0.10$, ~ : $p < 0.10$, * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$, **** : $p < 0.0001$.

Unfiltered Knotweed Rhizome Extract (KRE)

Filtered Knotweed Rhizome Extract (KRE)

Legend:
- x < −0.30
- −0.30 < x < −0.20
- −0.20 < x < −0.10
- −0.10 < x < −0.05
- −0.05 < x < 0.05
- 0.05 < x < 0.10
- 0.10 < x < 0.20
- 0.20 < x < 0.30
- x > 0.30

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Figure 5 : Multigroup path model of soil mesofaunal food webs after filtered or unfiltered knotweed rhizome addition. Differences between the

observed multigroup model and a "null model" with fixed Intercepts and Regressions was assessed with an ANOVA. Green arrows indicate a

772 positive correlation while red arrows indicate a negative correlation. Arrow width is proportional to the strength of the relationship. KRE concent. =

773 knotweed rhizome extract concentration level, Fungi = ergosterol concentration, Microbial Biomass = carbon amount in microbial biomass, Fungiv.

774 nemat. = Fungivorous nematodes abundance, Bacter. nemat. = Bacterivorous nematodes abundance, Predat. nemato. = Predatorous nematodes

775 abundance, Herb.-Fung. Acari = Herbo-fungivorous Acari, Predat. Acari = Predatorous Acari, Collemb.= Collembola.

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779 Tables

780

781 Table 1: Relative differences in physico-chemical and microbiological variables compared to control values (%) between knotweed rhizome extract

782 exposed pots and control pots. Values are means +/- SE. P-values are from repeated Wilcoxon rank-sum tests on absolute relative differences.

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Percentage differences	33% *v/v*	66% *v/v*	100% *v/v*

from control

	Combined	Nutrient	Secondary	Combined	Nutrient	Secondary	Combined	Nutrient	Secondary
Ergosterol content	8.05 ± 6.97	1.88 ± 4.69	0.06 ± 0.07	4.75 ± 4.97	8.99 ± 4.99	-3.89 ± 4.56	3.86 ± 4.26	0.11 ± 7.16	3.75 ± 4.25
	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Microbial carbon	-4.2 ± 6.11	-4.29 ± 6.18	0 ± 0.06	-7.78 ± 5.91	-10.74 ± 4.04	3.32 ± 6.63	-5.31 ± 6.55	-11.38 ± 8.87	6.84 ± 7.39
	n.s.	n.s.	n.s.	n.s.	$p < 0.05$	n.s.	n.s.	n.s.	n.s.
Ergosterol / Microbial C	10.98 ± 11.32	3.98 ± 7.48	0.07 ± 0.11	10.81 ± 7.52	17.25 ± 6.06	-5.5 ± 6.42	9.17 ± 9.67	24.16 ± 22.81	-12.07 ± 7.79
	n.s.	n.s.	n.s.	n.s.	$p < 0.05$	n.s.	n.s.	n.s.	n.s.
pH (KCl)	-1.05 ± 0.48	1.39 ± 0.4	-0.02 ± 0	0.93 ± 0.46	0.39 ± 0.43	0.53 ± 0.46	0.48 ± 0.49	-0.89 ± 0.29	1.38 ± 0.5
	$p < 0.10$	$p < 0.01$	$p < 0.05$	$p < 0.10$	n.s.	n.s.	n.s.	$p < 0.05$	$p < 0.05$
C/N ratio	-0.18 ± 0.55	-0.52 ± 0.49	0 ± 0.01	0.11 ± 0.67	0.64 ± 0.6	-0.52 ± 0.67	-1.37 ± 0.56	-0.07 ± 0.57	-1.3 ± 0.57
	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	$p < 0.05$	n.s.	$p < 0.10$

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788 Table 2: Relative differences in nematode abundances between knotweed rhizome extract exposed pots and control pots. Values are means

789 percentages of difference +/- SE. P-values are from repeated Wilcoxon rank-sum tests.

Percentage differences from control	33% *v/v*			66% *v/v*			100% *v/v*		
	Combined	Nutrient	Secondary	Combined	Nutrient	Secondary	Combined	Nutrient	Secondary
Bacterivores	-43.52 ± 20.78	59 ± 34.27	-0.64 ± 0.13	-27.64 ± 15.05	54.43 ± 25.03	-53.14 ± 9.75	58.3 ± 21.49	12.19 ± 36.48	41.11 ± 19.15

	p < 0.10	n.s.	n.s.	n.s.	p < 0.10	p < 0.001	p < 0.05	n.s.	p < 0.10
Herbivores	16.12 ± 23.1	69.23 ± 32.53	-0.31 ± 0.14	43.93 ± 33.55	66.01 ± 38.26	-13.3 ± 20.21	177.12 ± 60.86	59.19 ± 21.25	74.09 ± 38.23
	n.s.	p < 0.10	n.s.	n.s.	n.s.	n.s.	p < 0.05	p < 0.05	p < 0.10
Fungivores	-79.83 ± 5.26	138.03 ± 43.19	-0.92 ± 0.02	-49.79 ± 12.07	6.67 ± 18.76	-52.93 ± 11.32	24.52 ± 28.52	-16.06 ± 18.97	48.35 ± 33.98
	p < 0.0001	p < 0.05	n.s.	p < 0.01	n.s.	p < 0.01	n.s.	n.s.	n.s.
Predators/omnivores	-18.82 ± 19.73	-39.64 ± 24.42	0.34 ± 0.33	-8.71 ± 28.14	42.23 ± 33.72	-35.81 ± 19.78	47.14 ± 35.15	34.69 ± 52.25	9.24 ± 26.1
	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

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795 Table 3: Relative differences in mesofauna (Collembola and Acari) abundance, taxonomic and functional indices between knotweed rhizome extract

796 exposed pots and control pots. Values are means +/- SE. P-values are from repeated Wilcoxon rank-sum tests.

Percentage differences from control	33% *v/v*			66% *v/v*			100% *v/v*		
	Combined	Nutrient	Secondary	Combined	Nutrient	Secondary	Combined	Nutrient	Secondary
Acari									
Gamasid mites	73.58 ± 30.4	24.53 ± 16.92	0.39 ± 0.24	133.96 ± 30.71	13.21 ± 20.28	106.67 ± 27.13	35.85 ± 28.79	13.21 ± 31.82	20 ± 25.43
	p < 0.05	n.s.	n.s.	p < 0.01	n.s.	p < 0.01	n.s.	n.s.	n.s.
Oribatid mites	203.85 ± 65.99	69.23 ± 39.39	0.8 ± 0.39	219.23 ± 71.16	142.31 ± 77.57	31.75 ± 29.37	415.38 ± 77.39	38.46 ± 53.85	272.22 ± 55.89

	$p < 0.05$	n.s.	n.s.	$p < 0.05$	$p < 0.10$	n.s.	$p < 0.001$	n.s.	$p < 0.001$
Collembola									
	n.s.	n.s.	n.s.	$p < 0.01$	$p < 0.10$	n.s.	$p < 0.05$	n.s.	$p < 0.10$
	57.54 ± 22.41	43.05 ± 28.93	0.1 ± 0.16	109.41 ± 20.98	51.9 ± 34.08	37.86 ± 13.81	19.82 ± 27.1	79.26 ± 21.71	-33.16 ± 15.12
Shannon's diversity									
	$p < 0.05$	n.s.	n.s.	$p < 0.001$	n.s.	$p < 0.05$	n.s.	$p < 0.01$	$p < 0.10$
	24.93 ± 34.62	15.61 ± 22.45	0.08 ± 0.3	70.38 ± 27.46	5 ± 32.7	62.26 ± 26.15	-40 ± 27.42	13.5 ± 29.19	-47.14 ± 24.15
Functional richness									
	n.s.	n.s.	n.s.	$p < 0.05$	n.s.	$p < 0.05$	n.s.	n.s.	$p < 0.10$
	-5.3 ± 4.13	-0.85 ± 4.7	-0.04 ± 0.04	-8.88 ± 3.46	1.26 ± 3.98	-10.02 ± 3.42	-1.31 ± 5.2	-1.61 ± 4.33	0.31 ± 5.28
Functional evenness									
	n.s.	n.s.	n.s.	$p < 0.05$	n.s.	$p < 0.05$	n.s.	n.s.	n.s.