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C. Abgrall, E. Forey, L. Mignot, M. Chauvat

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1 Invasion by *Fallopia japonica* alters soil food webs through secondary metabolites

2

3 Abgrall, C.^{a,b}; Forey, E.^a; Mignot, L.^a; Chauvat, M.^a

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5

6 ^a Ecodiv URA/EA 1293, IRSTEA, FR CNRS 3730 SCALE, UFR Sciences et Techniques,

7 Normandie université, Université de Rouen, 76821 Mont Saint Aignan Cedex, France

8 ^b Corresponding author ; corentin.abgrall1@univ-rouen.fr

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

42 Keywords

43 plant-soil interactions ; novel weapons hypothesis ; allelopathy ; trophic networks ; alien
44 species

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. mycorrhizous 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 ($4 \times 2 \times 10 = 80$ 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 ($21.0 \pm 1.9^\circ\text{C}$, 16h day / 8h night, $47.0 \pm 8.8\%$ 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 ($\text{C}_{14}\text{H}_{12}\text{O}_3$), 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 $1 \text{ ml}\cdot\text{min}^{-1}$ (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 empirical model using ANOVA. This approach provides a means to
235 assess for covarying 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 ($-10.74 \pm 4.04\%$; $p < 0.05$; 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 ($-0.02 \pm 0\%$; $p < 0.05$; Tab. 1) which shifted
250 to an increase at the highest concentration ($+1.38 \pm 0.5$; $p < 0.05$; Tab. 1). The C/N ratio
251 remained largely unaffected by KRE input except at the highest concentration (combined
252 effect: $-1.37 \pm 0.56\%$; $p < 0.05$; Tab. 1).

253 Resveratrol concentration for 100% v/v KRE was $2.27 \pm 0.23 \text{ mg.l}^{-1}$ 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 ($+64.8 \pm 17.6$ %) which was only
279 significant at the highest concentration ($+59.2 \pm 21.3$ %; 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 insignificant, effect of nutrient
297 addition seems to exist at the intermediate concentration ($+56.9 \pm 28.4$ %; $p < 0.10$; 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) ($n_{group1} = 40$, $n_{group2} = 40$, Chi^2 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 insignificant in all cases for both fungal and microbial
380 biomass (Tab. 1). Hence, while there may be concentration-dependence the effects
381 themselves are insignificant, 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 detritivores). 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. Hedeneč 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 insignificantly, 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 shifts 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 insignificant 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 significant 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 insignificantly 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

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523

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532 7. References

533

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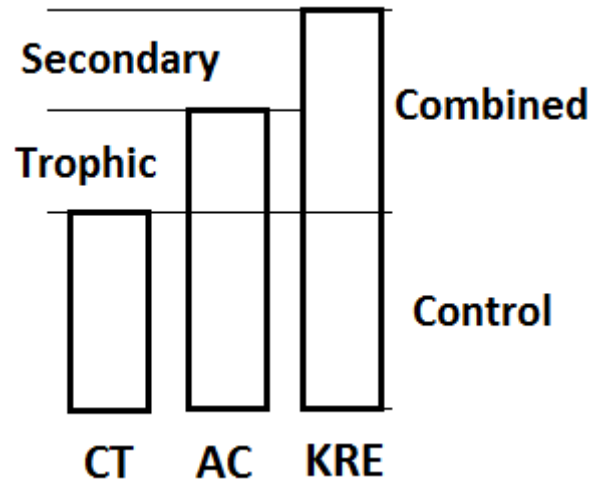
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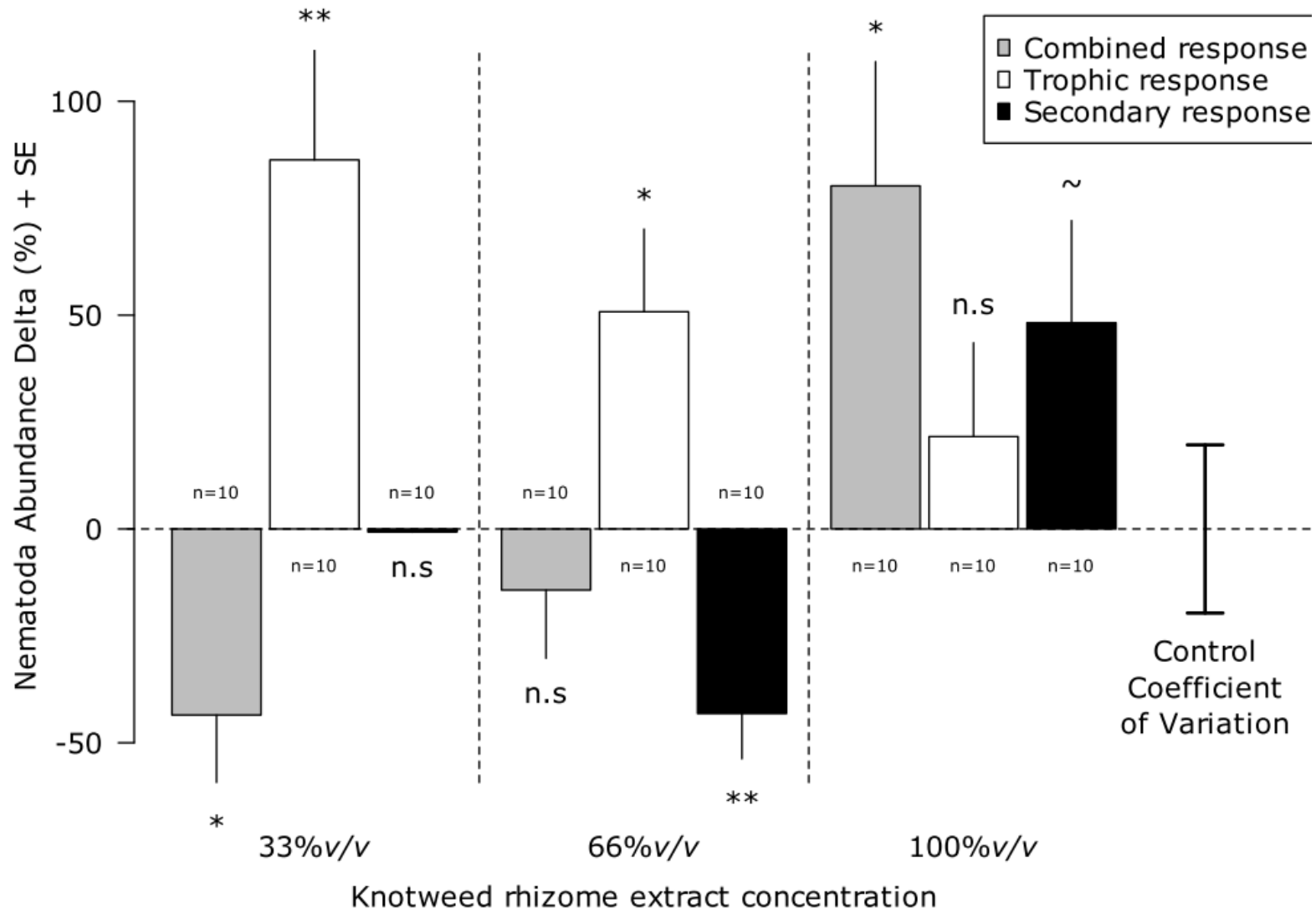
753 Figures



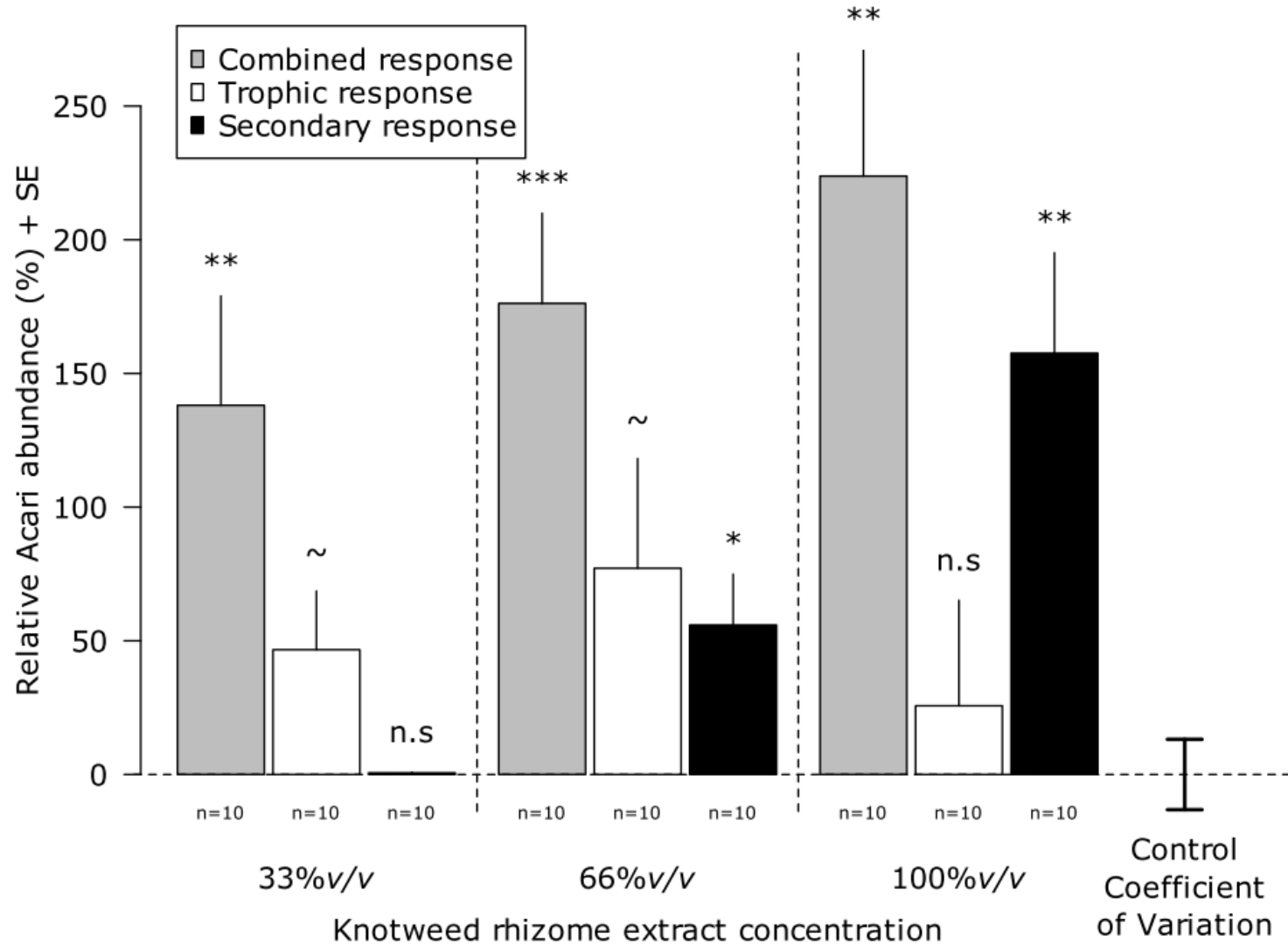
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755 Figure 1 : Diagram of calculations involved in separating knotweed rhizome extract (KRE) effects. CT: control, AC: activated carbon filtration, KRE:

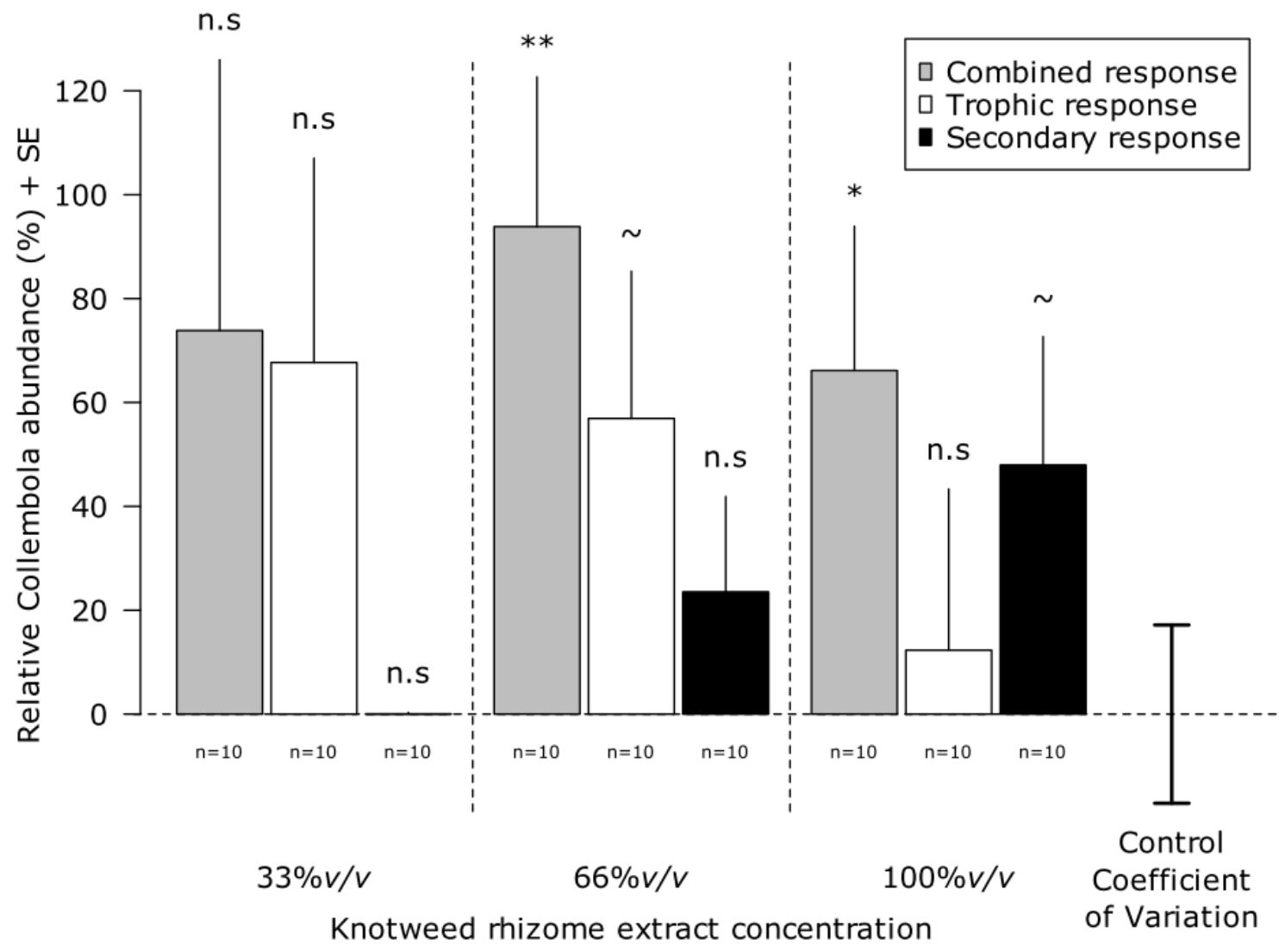
756 no AC filtration.



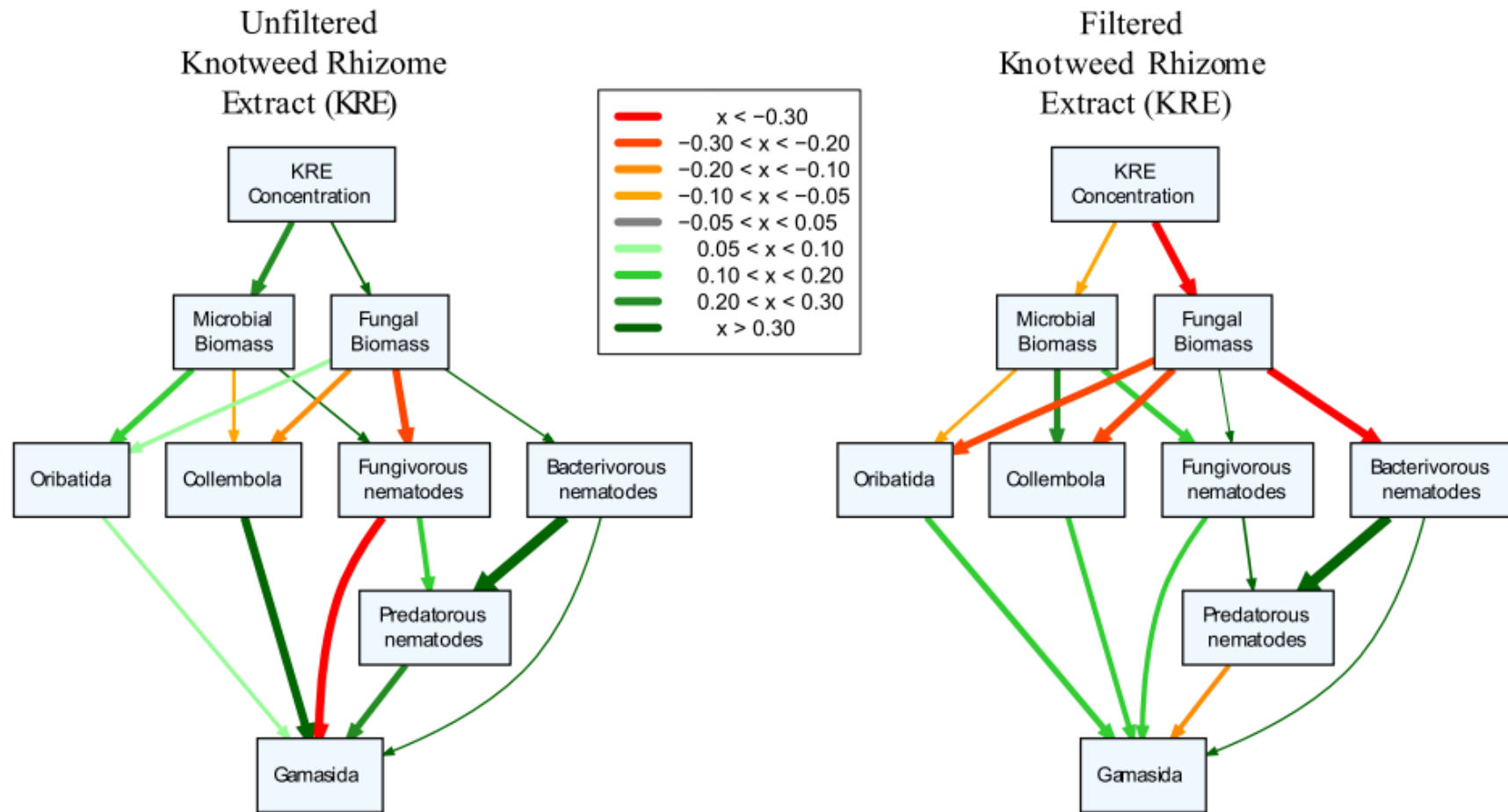
758 Figure 2 : Relative nematode total abundance (%) compared to control in relation to knotweed rhizome extract dilution levels and activated carbon
759 filtration with decomposition of effects. Symbols indicate levels of significativity of repeated statistical testing of differences between calculated
760 values and null generated controls. n.s.: $p > 0.10$, ~ : $p < 0.10$, * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$, **** : $p < 0.0001$.



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.: $p > 0.10$, ~ : $p < 0.10$, * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$, **** : $p < 0.0001$.



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.: $p > 0.10$, ~ : $p < 0.10$, * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$, **** : $p < 0.0001$.



769

770 Figure 5 : Multigroup path model of soil mesofaunal food webs after filtered or unfiltered knotweed rhizome addition. Differences between the
 771 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.

783

Percentage

33% v/v

66% v/v

100% v/v

differences

from control

	Combined	Nutrient	Secondary	Combined	Nutrient	Secondary	Combined	Nutrient	Secondary
Ergosterol	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
content	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Microbial	-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
carbon	n.s.	n.s.	n.s.	n.s.	p < 0.05	n.s.	n.s.	n.s.	n.s.
Ergosterol /	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
Microbial C	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

33% v/v

66% v/v

100% v/v

from control

Combined

Nutrient

Secondary

Combined

Nutrient

Secondary

Combined

Nutrient

Secondary

Bacterivores

-43.52 ±

-27.64 ±

20.78

59 ± 34.27

-0.64 ± 0.13

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
							177.12 ±		
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	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
		138.03 ±		-49.79 ±		-52.93 ±		-16.06 ±	
Fungivores	-79.83 ± 5.26	43.19	-0.92 ± 0.02	12.07	6.67 ± 18.76	11.32	24.52 ± 28.52	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.
	-18.82 ±	-39.64 ±				-35.81 ±			
Predators/omnivores	19.73	24.42	0.34 ± 0.33	-8.71 ± 28.14	42.23 ± 33.72	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		24.53 ±		133.96 ±		106.67 ±		13.21 ±	
	73.58 ± 30.4		0.39 ± 0.24		13.21 ± 20.28		35.85 ± 28.79		20 ± 25.43
Gamasid mites		16.92		30.71		27.13		31.82	
	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 ±	69.23 ±		219.23 ±	142.31 ±		415.38 ±	38.46 ±	272.22 ±
	65.99	39.39	0.8 ± 0.39	71.16	77.57	31.75 ± 29.37	77.39	53.85	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
		43.05 ±		109.41 ±				79.26 ±	-33.16 ±
Shannon's diversity	57.54 ± 22.41	28.93	0.1 ± 0.16	20.98	51.9 ± 34.08	37.86 ± 13.81	19.82 ± 27.1	21.71	15.12
	p < 0.05	n.s.	n.s.	p < 0.001	n.s.	p < 0.05	n.s.	p < 0.01	p < 0.10
		15.61 ±		70.38 ± 27.46	5 ± 32.7	62.26 ± 26.15	-40 ± 27.42	13.5 ± 29.19	-47.14 ±
Functional richness	24.93 ± 34.62	22.45	0.08 ± 0.3						24.15
	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 ±	-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			0.04						
	n.s.	n.s.	n.s.	p < 0.05	n.s.	p < 0.05	n.s.	n.s.	n.s.