Invasion by Fallopia japonica alters soil food webs through secondary metabolites

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Abstract

Biological invasions are a major threat to biodiversity with varying degrees of impact. There is increasing evidence that allelopathy often plays an important role in explaining both invasion success and impact on native taxa (e.g. novel weapons hypothesis). The effects of these secondary metabolites on plant communities and microorganisms are well known. However, their direct and indirect effects on soil fauna are unresolved, despite the importance of the latter in ecosystem processes and, potentially, invasion mitigation. Japanese knotweed (*Fallopia japonica*), an east-Asian species, which has proved to be invasive in Europe, containing allelopathic secondary compounds inhibiting native plants and microbial communities. The focal point of this study was the allelopathic effects of knotweed on soil mesofauna (Nematoda, Collembola and Acari). During a one-month laboratory experiment we added knotweed rhizome extract (KRE) at different concentrations to soils collected in an invasion-prone area. He experiment consisted of

including or excluding secondary metabolites through the use of activated carbon filtration of KRE. This enabled us to separate effects caused by nutrient addition (i.e. trophic effects) and combined (trophic and allelopathic) effects. Relative effects of nutrient and secondary metabolites addition on abiotic and biotic soil variables were then quantified. We highlighted frequently contrasting trophic and allelopathic effects influenced in some cases by KRE concentration. Microbial assemblages, through fungal / microbial biomass ratio, did not show any congruent response to KRE secondary compounds but was more negatively impacted by nutrient addition. The use of a trophic-based path analysis led us to show that changes within the soil biota had repercussions on secondary consumers (e.g. bacterivorous nematodes and Collembola). Abundance within taxa at higher trophic levels such as predatory Acari (but not predatory nematodes) was also affected although to a lesser extent, likely in part due to the limited considered timeframe. Overall, we showed that, in controlled conditions, invasive allelopathic plants such as knotweeds can have effects on soil fauna at different trophic levels through addition of both nutrients and secondary metabolites to the soil. Considering the limited knowledge of allelopathic effects on the soil fauna and soil functions, this study provides new information on above- and belowground interactions.

Keywords

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

1. Introduction

Past and current introduction of invasive plant species and their spread in new ecosystems is a major concern for conservation at a global level (Litt et al., 2014; Pyšek et al., 2012) due to their severe impact on biodiversity (Murrell et al., 2011; Vilà et al., 2011) and ecosystem processes (Bassett et al., 2011; Kohyt and Skubała, 2013). Only a small number of exotic species become invasive in their introduced range (Reinhart and Callaway, 2006) through distinctive characteristics (or traits) providing superior competitive ability when compared to native species (Van Kleunen et al., 2010). These traits can be morphological in nature by directly improving plant fitness (Van Kleunen et al., 2010) or physiological with the synthesis of biochemical, secondary metabolites that influence the germination, growth, survival and/or reproduction of other organisms (Inderjit et al., 2011b).

The novel weapon hypothesis (NVH) suggests that the success of many exotic invasive plant species is due to the possession of allelopathic compounds unencountered by native species, particularly native plant species (Callaway and Ridenour, 2004). Furthermore, it has been shown that many invasive species have different allelopathic potential effects between their native and introduced ranges (Inderjit et al., 2011a; Thorpe et al., 2009). These biochemical compounds, exudated from plant roots (Callaway et al., 2008) or released from degrading litter (Inderjit et al., 2011a) have powerful effects on ecosystem functioning by impacting both organisms and ecological processes (Hättenschwiler et al., 2011; Hättenschwiler and Vitousek, 2000; Reigosa et al., 2006; Wardle et al., 1998).

Under the soil, plants interact with a wide range of organisms including bacteria, fungi, nematodes and various kinds of arthropods (Abgrall et al., 2017; Parepa et al., 2013). These aboveground-belowground relationships can be antagonistic (e.g. herbivores, pathogens) or mutualistic (e.g. mycorrhizeous fungi, nitrogen-fixing bacteria) (Van der Putten et al., 2007). Allelopathic biochemical that have a negative effect on plants can do so indirectly by 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

indirect effect through changes in the soil biota (Ens et al., 2009). As evidence for direct toxicity of phenolic compounds is scarce, indirect effects through alterations of basal resources for secondary consumers appear more likely. In this paper, we studied the effect of knotweed rhizome aqueous extracts on the soil biota and fauna in order to provide additional information on the novel weapon hypothesis in this particular case. Indeed, while several studies have assessed knotweed allelopathic potential in invaded areas none, as far as we know, have considered the impact on the soil fauna in relation to this hypothesis. Therefore and based on the theory, we hypothesized that: (1) knotweed has a negative effect on microbial (and particularly bacterial) biomass through rhizome allelopathic secondary metabolites ; (2) this negative effect has repercussions on higher trophic levels through trophic cascades, and results in soil food web structure alteration ; (3) this negative effect is be slightly attenuated by a positive trophic effect of nutrient addition provided by knotweed rhizome extract ; (4) those effects, positive (i.e. trophic) or negative (i.e. allelopathic), are concentration-dependent.

2. Material & Methods

2.1. Material collection and experiment preparation

Belowground *F. japonica* biomass was harvested in early autumn 2016 within a spontaneously invaded plateau site in Normandy, France (49.455024° N; 1.062645° W). To the best of our knowledge, control measures have never been applied to this site. Samples were kept in an icebox for transportation to the laboratory. Rhizomes were water-cleaned and stored at 4°C prior to extraction. We used an electric grinder to break plant tissues and

facilitate osmosis. One hundred grams of ground plant material was mixed with 1000 ml of distilled water. This aqueous extract was kept at 19°C for 24h. Following Norsworthy (2003) the mixture was then passed through a series of sieves ranging from 1000 to 50 µm and then vacuum filtered through standard filter paper (> 20-25 µm). The extract was then further sterilized by filtering through 0.22 µm filter.

We collected soil from the upper 10 cm of a reaped grassland in a small valley. While the area was uninfested by *F. japonica* close-by sites (< 200 m) with similar topographical and edaphic conditions have been invaded for several years. Macrofauna as well as macroscopic plant materials were removed from the collected soil. Samples were gently, and unforcefully, sieved at 5 mm so as to preserve mesofauna and mixed. Ten 200 g samples were taken from the soil to sample initial Collembola, Acari and Nematoda communities. Soils were placed in 8 x 8 x 10 cm plastic pots. Filter paper (<10-20 µm) was placed at the bottom of the prevent leakage of the pot content. Sixty grams of fine grained sand was added above the filter paper forming a ~ 0.5 cm layer. The rest of the pot was filled with 310 ± 5 g of soil. Ten 400 g samples of mixed soil were also collected for analysis of initial physico-chemical conditions. A layer of 0.5 g of *Agropyron* sp. litter, which is the dominant species in the samples grassland and also present close to invaded sites, was added to provide physical habitat for the soil fauna. Pots were kept at 19°C in a phytotron with a 8h day / 16h night cycle for a week. In order to increase and homogenize abundance and compensate for possible losses during soil sieving each pot was then placed under 2 individual Berlese-Tullgren extractors, one containing topsoil (0 – 5 cm) and the other deeper soil (5 – 10 cm) from the same area.

2.2. Experimental design

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,

diameter: 80 mm, height: 50 mm) above a plastic funnel. Extraction, under a heat source, lasted for a week with individuals collected in 70% ethanol. This extraction method is dependent on the limited tolerance of these animals to desiccation and will therefore only extract active individuals. There is therefore no differentiation between individuals that were inactivated, killed or otherwise incapacitated. One hundred grams of fresh soil was used for nematode extraction in a Baermann funnel (McSorley and Walter, 1991). Dampened samples were placed in a porous paper (10-15 μm stitch) supported by a 2 mm sieve and placed above a water-filled and sealed funnel for 48h. This method has a limited efficiency in isolating slow moving and nematodes and will not isolate inactive individuals (Van Bezooijen, 2006) and thus is not exhaustive.

Mesofauna samples were separated into Acari and Collembola under a stereo binocular microscope. Collembola individuals were mounted in lactic acid on microscope slides for identification with a phase-contrast optical microscope. Collembola individuals were identified to the species level (Hopkin, 2007; Potapov, 2001; Thibaud, 2004). Acari were identified to the order or suborder level: Mesostigmata (Gamasida), Cryptostigmata (Oribatida) and Prostigmata (Actinedida) (Coineau and Cleva, 1997). The cohort Astigmatina (previously the suborder Astigmata) were included in the suborder Oribatida (Wang and Fan, 2010). After Baermann funnel extraction, nematodes were counted while active under a stereo binocular microscope. Following decantation nematodes were fixed using a 4% formaldehyde solution and mounted on microscope slides. Individuals were attributed to trophic groups (herbivores, bacterivores, fungivores and predators/omnivores) based on mouthpart examination under a compound optical microscope.

Four grams of fresh soil were used to measure soil ergosterol content using the method proposed by Gong, Guan & Witter (2001). Ergosterol is a sterol found within fungi and protozoa. Ergosterol concentration can be used as a proxy of soil fungal biomass. Two times

20 g of fresh soil was used to assess microbial biomass using chloroform fumigation and extraction (Brookes et al., 1985). Carbon extraction was performed in 100 ml of potassium sulphate 0.05M (K_2SO_4) for chloroform-fumigated or unfumigated samples.

Once springtail extraction was complete a 30 g dry soil aliquot used to assess soil abiotic variables. Soil pH was measured using 1:5 volumetric fraction in 1M potassium chloride (KCl) using a Mettler Toledo FiveEasy pH meter. Total carbon and nitrogen content was measured in a ThermoFisher Flash Analyzer 2000 after electric grinding of dry soil material.

2.4. Statistical analysis

We used ANOVA tests included in R Software 3.3.1 for statistical analysis. In order to assess for knotweed rhizome extracts (KRE) effect on the soil fauna and microbiology we calculated the relative differences between the considered modalities and our control by standardizing and normalizing our measured values in relation to control means. This was done by subtracting the average control value to each value for the considered modality. Based on our methodology and hypotheses we considered that several of the potential effects of KRE could be separated. We thus considered that subtracting the control mean to values obtained in modalities where KRE was unfiltered by activated carbon (AC) values gave the combined effect of KRE on the considered variable (Fig. 1). Based on the generally accepted hypothesis we considered that AC filtration retains knotweed secondary compounds. Therefore, subtracting control means to values measured in pots subjected to AC filtered KRE we calculated KRE effect not attributable to secondary metabolites (Fig. 1). We referred to this effect as a trophic effect that could directly affect soil microbiology by adding nutrients to the substrate with potential indirect repercussions on higher trophic levels.

Finally, the same methodology was used by subtracting for each concentration the mean AC filtered value to individual values measured in pots that received unfiltered KRE.

We used a null model approach in order to consider control stochastic variability. Null vectors were randomly generated for each variable based on the observed distribution parameters of the control. Significant differences between these null vectors and our data vectors was assessed using non-parametric Wilcoxon signed-rank test. An iterative procedure (one-thousand repetitions) and result aggregation enabled us to robustly, and conservatively, assess for statistical differences between control and treatments. These differences, when found, showed KRE effects on the considered variables.

We also assessed for differences in the structure of the soil food web with and without AC filtration, and thus with or without knotweed secondary metabolites. We used multigroup path analysis to model our empirically observed model and compare it to a "null" multigroup model. The "null" model had constrained intercepts and regression coefficients that was compared to the empiracl model using ANOVA. This approach provides a means to assess for covariating responses of soil fauna compartments to potential allelopathy.

3. Results

3.1. Physico-chemistry & microbiology

Contrary to our hypothesis and the literature, we observed limited effect of knotweed rhizome extracts (KRE) on microbiological variables. There were no significant differences in ergosterol concentration, an indicator of fungal biomass, irrespective of concentration or 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

compounds addition (from -43 % at intermediate concentration to +48 % at maximum concentration; Fig. 2; Tab. 2).

Although significance and intensity differed, bacterivorous and fungivorous nematodes (41.07 ± 1.59 % and 22.35 ± 1.26 % of total nematodes abundance, respectively) response to KRE addition varied similarly to the general trend with a shift from a negative (-43.5 % / -79.8 %) to a positive (+58.3 % / +24.5 %) response with increasing KRE concentration (Tab. 2). Herbivorous nematodes abundance (28.0 ± 1.3 % of total abundance) varied somewhat differently with a significant, and positive, response to KRE addition only at the highest concentration (+177 % increase; Tab. 2). Nutrient addition appeared to elicit a generally positive response independently of concentration (+64.8 ± 17.6 %) which was only significant at the highest concentration (+59.2 ± 21.3 %; Tab. 2) while there was no significant response to secondary compounds addition. Predatorous and omnivorous nematodes showed no significant response to KRE addition (Tab. 2).

3.3. Mesofauna

Total Acari relative abundance showed a strong positive response to KRE input at all concentrations without significant differences in intensity (from +138.1 to +223.8 % as concentration increased; Fig. 3) which appears to me mostly related to a response to secondary compounds addition. A similar pattern was observed for oribatid mites Tab. 3). There were significant differences between responses at the lowest and highest concentration levels for both the combined and secondary compounds responses but not for response to nutrient addition (Tab.3). Predators (i.e. mostly Gamasida) abundances responded only to the combined aspects of KRE addition, and only at the two lowest 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) ($n_{group1} = 40$, $n_{group2} = 40$, 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

(0.126 p = 0.000. to 0.111 p = 0.000; Fig. 5). The relationship between herbo-fungivorous and predatorous Acari abundance was not significantly altered by AC filtration (0.030 p = 0.593. to 0.089 p = 0.176; Fig. 5).

Concerning the fungal pathway pathway allelopathic effect removal through activated carbon (AC) filtration decreased the relationship, yet with still no significant relationship, between KRE concentration and fungal biomass (i.e. ergosterol concentration) (0.013 $^{p = 0.174.}$ to -0.007 $^{p = 0.571}$; Fig. 5). Fungal biomass relationships with its consumers was also affected by allelopathic effect removal: with herbo-fungivorous Acari (18.681 $^{p = 0.166.}$ to -3.119 $^{p = 0.693}$; Fig. 5), Collembola abundance (-1.464 $^{p = 0.928.}$ to 17.470 $^{p = 0.118}$; Fig. 5) and, in a very limited way, fungivorous nematodes abundance (-1.973 $^{p = 0.836.}$ to 8.794 $^{p = 0.274}$; Fig. 5). Strengths of relationships of these taxa with their predators also changed after allelopathic effect removal especially fungivorous nematodes (-0.213 $^{p = 0.011}$ to -0.067 $^{p = 0.348}$; Fig. 5) and Collembola (0.126 $^{p = 0.009}$ to -0.050 $^{p = 0.282}$; Fig. 5) with predatorous Acari. The relationship between fungivorous and predatorous nematodes abundance was mostly unaffected (-0.030 $^{p = 0.593}$ to 0.089 $^{p = 0.176}$; Fig. 5) as was the relationship between the two main predatorous groups (i.e. predatorous nematodes and Acari) (0.244 $^{p = 0.161}$ to -0.119 $^{p = 0.496}$; Fig. 5).

4. Discussion

The first hypothesis posited a negative effect of knotweed allelopathic secondary compounds (ASC) on microbial communities as generally observed *in situ* (Hedenec et al., 2014; Tamura and Tharayil, 2014). The effect of ASC addition was not directly tested on the soil fauna, due in part to the lack of a proper identification of all such potential compounds in knotweed (Fan et al., 2010). However, we were able to ascertain the removal of ASC from

a solution of knotweed rhizome extract (KRE) by using activated carbon which is known to suppress allelopathic effects (Ridenour & Callaway, 2001 but see Lau et al., 2008 for a critic of this methodology). We were able to test and demonstrate removal of one ASC, trans-resveratrol, from KRE through activated carbon filtration (see Supplementary Material A1). Differences in population responses between activated carbon filtered and unfiltered KRE addition to the soil was therefore considered to be mainly, but not only, due to removal of ASC. We then indirectly calculated a "secondary" effect of KRE. Contrary to our hypothesis, we generally did not find any significant antimicrobial effects on ergosterol concentration (a proxy of fungal biomass; Davis & Lamar, 1992), microbial carbon (a proxy of microbial biomass; Vance et al., 1987) or the ratio between the two (a rough indicator of microbial community structure; Djajakirana et al., 1996; Tab. 2). Ergosterol concentration itself remained unaffected by KRE at concentrations which is consistent with the contrasting, yet often positive, effects found in the literature on the effects on fungal biomass (Daayf et al., 1995; Lecerf et al., 2007; Tamura and Tharayil, 2014). The pro-microbial, albeit insignificant, effects are far more surprising and tend to refute our hypothesis and contrast with results found in the literature (Hedenec et al., 2014; Kumagai et al., 2005; Stefanowicz et al., 2016; Tamura and Tharayil, 2014). Most of these results were observed in the field, with multiple potential confounding factors, with only Daayf et al., 1995 and Kumagai et al., 2005 directly testing antimicrobial and antifungal properties of knotweeds secondary compounds in controlled conditions. For instance, a major source of knotweed allelopathic properties are linked to the slow degradation and release of phenolic compounds from leaf litter degradation (Lavoie, 2017) which we did not account for in this study. Overall while the antibacterial effect of knotweeds in general, and Japanese knotweed in particular, appear fairly conclusive in the literature our results tend to show that this cannot be attributed to, or only to, rhizome secondary metabolites.

The final hypothesis stipulated that knotweed KRE-addition effects, in particular ASC addition, were linearly concentration-dependent. This hypothesis cannot be properly segregated from the other and will be considered here to avoid repetition. This hypothesis was based on the literature which frequently mentions release of secondary metabolites in the environment by knotweeds as a major contributor to knotweed effects in their invasive range (Vastano et al., 2000). Other lab studies have shown concentration-dependent effects of some root-secreted phenolic compounds on microbial biomass (e.g. Zhang et al., 2015). These compounds; however, are not in the same family as resveratrol or catechin. We tested this hypothesis indirectly as the effect of secondary metabolites was calculated and not measured. Concerning the concentration-dependence of microbiological response to ASC the relationship remained unsignificant in all cases for both fungal and microbial biomass (Tab. 1). Hence, while there may be concentration-dependence the effects themselves are unsignificant, and we must therefore accept the alternative hypothesis in the case of ASC. Nutrient addition, however, significantly and negatively affected microbial biomass at 66% v/v concentration only, showing differences dependent on concentration. However, as this is for intermediate concentration there does not appear to be a linear relationship between concentration and response. When analyzing changes in soil food web structure we showed that allelopathic secondary compounds (ASC) removal increased the strength of the negative correlation between KRE concentration and microbial biomass, the corollary being that ASC addition would tend to decrease the strength of the relationship between the two (Fig. 5). Only after ASC removal was the relationship significant between the two variables. Therefore, while the combined, and ASC, effects on microbial biomass appear not to be concentration-dependent purely “trophic” effects are significantly so.

The second hypothesis stated that, if there were antimicrobial and antifungal effects of KRE, they would have repercussions on higher trophic levels through a trophic cascade with potential alterations of trophic structures. We showed previously that no antimicrobial or

antifungal effect were found in our experiment in response to KRE of allelopathic secondary compounds (ASC). However, the evidence revealed significant differences in abundance at higher trophic levels. Indeed, we did find significant effects of KRE on bacterivorous and fungivorous nematodes (both positive and negative) abundances as well as herbo-fungivorous Acari and Collembola abundances (mostly positive) from both allelopathic or combined effects (although with no consistent pattern at different concentrations; see Tab 2-3). We found no significant differences in predator nematodes abundances despite the changes in abundance of their prey. Predator Acari, on the other hand, had higher abundances following KRE addition. Accordingly, predators may have had a top-down effect on microbivores while being unaffected themselves by bottom-up regulation themselves in the considered timeframe. Unfortunately, we could not find any reports in the literature on nematodes abundances under knotweed and therefore cannot assess the representativity of our results. Skubala & Mierny (2009), on the other hand, found a significant negative effect of giant knotweed (*Fallopia sachalinensis*) on oribatid mites (mostly herbivores, fungivores or both) but no effect on Collembola abundance (mostly generalist fungivores and detrivores). They attributed observed effects in the field on liberation phenolic compounds from leaf litter degradation, not rhizome excretion. This is, to our knowledge, the only publication to date assessing knotweed effects on the soil mesofauna in spontaneously invaded sites. Hedenec et al. (2014) also considered Collembola and Acari, with mixed results and no differences with native species, but in an agricultural setting with giant knotweed used as a biofuel crop. Macroarthropod abundance, which we did not consider in this study, has generally been shown to be negatively affected by knotweed presence (Gerber et al., 2008; Kappes et al., 2007; Topp et al., 2008)

Collembola and Acari have a generation time of several weeks to months, depending on taxa and eco-morphological group (Choi et al., 2002; Ermilov and Lochynska, 2008; Joosse and Veltkamp, 1969; Park, 2007; Prinzing et al., 2002; Verhoef and Selm, 1983). Observed

differences are thus unlikely to be caused by a predator-prey intergenerational regulation but would be due to a more direct effect. Predatory nematodes and Acari have generation times ranging from 3 to 280 days depending on taxa (Abou-Awad et al., 2001; Khan et al., 2007; Ydergaard et al., 1997) as well as temperature. For some taxa within our study the experiment duration, 1 month, may have been insufficient to observe significant repercussions on higher trophic levels through intergenerational predator-prey relationships.

We also assessed how activated carbon filtration, and thus ASC removal, affected the structure of the soil trophic network by using multigroup path analysis (Fig. 5). The comparison of an empirical model to a constrained model, clearly showed that ASC removal significantly altered overall relationships between the various considered faunal groups. In this analysis, ASC removal also seemed to more readily alter relationships between taxa in the microbial food web (i.e. between microbial biomass and abundances of Collembola, oribatid mites and bacterivorous nematodes). This relationship was, however, negative. This would suggest that ASC removal from added KRE increases the interdependency of compartments/taxa within the microbial food web as well as the effect of KRE concentration on microbial biomass. The corollary, although we did not test it directly, would be that the allelopathic component of knotweed effect tends to limit between-group variability. In addition, it would appear that while nutrient input has concentration-dependent effects (at least on microbial biomass), nutrient and allelopathic effects are not dependent on concentration. Another striking feature is the change in the relationship between fungivorous nematodes and predatorous Acari, for which intensity was drastically reduced by ASC removal from KRE and most importantly switched from a negative to a positive correlation.

We also posited that part of knotweeds success as invaders was due to lack of adaptation by native species to the invader: the novel weapon hypothesis (Callaway and Ridenour, 2004). This framework most generally applies to other plant species with which there is more direct competition. While our methodology does not enable us to assess that effect, we expected negative effects (on abundance and diversity) of knotweed allelopathy on at least some taxa within the soil fauna which would help explain results found in several field studies (Gerber et al., 2008; Kappes et al., 2007; Skubala and Mierny, 2009; Topp et al., 2008). Such negative effects could have been direct through phytotoxicity or indirect through a trophic cascade or changes in habitat structure. This was not the case here with few negative responses of soil fauna to ASC addition. The only significant negative effects documented were for all types of nematodes in response to 66% KRE addition (Tab. 2).

The third hypothesis centered on the posited countering of negative allelopathic effects of KRE addition by nutrient addition. This appears to be the case, albeit unsignificantly, for the ergosterol / microbial C ratio at the two highest concentrations which is negatively affected by the allelopathic component of KRE (i.e. favoring microbial biomass) and positively affected by the nutrient-addition component (i.e. favoring fungal biomass) with a combined positive effect. Field studies evaluating fungal:bacterial ratios have found conflicting results on that matter with both increased (Suseela et al., 2016; Tamura and Tharayil, 2014) and decreased (Stefanowicz et al., 2016) ratios under knotweed-invaded plots. The results tend to support the first case of decreased bacterial biomass, although fungal biomass remained unaffected (Tab. 1). These effects of knotweed are often attributed to increased litter biomass (e.g. Suseela et al., 2016) and changes in litter chemistry (higher litter C/N ratios: Dassonville, Guillaumaud, Piola, Meerts, & Poly, 2011; Mincheva et al., 2014; Urgenson, Reichard, & Halpern, 2009; higher litter lignin content: Aguilera et al., 2010) in addition to the already mentioned allelopathic effects. Our decomposition of effects between nutrient and secondary compounds addition tends to indicate that this positive effect on

fungal:microbial ratios is mainly attributed to a positive response of microbial biomass to increased nutrient input to the soil. Due to lack of leaf litter this is not comparable to field nutrient input, but nonetheless worth considering. Secondary compounds, which can be twice as concentrated in knotweed-invaded plots (Suseela et al., 2016), appear to have a negative effect on fungi:microbial biomass ratios in this case. Changes in microbial biomass carbon have the most influence over shits in this ratio in our case (i.e. negative effect of nutrient-addition and positive effect of secondary compounds addition) which would to be contrary to our hypothesis. The pH responded in a similar manner with a significant negative nutrient addition effect, a significant positive secondary compounds effect but an unsignificant slightly positive combined effect. A decrease in soil pH has generally been observed in field studies (Dassonville et al., 2011, 2008; Kappes et al., 2007) and is also attributed to increased litter biomass in knotweed stands. This is, however, not always the case (Stefanowicz et al., 2017; Stoll et al., 2012)

Finally the results regarding microarthropod abundances (i.e. Collembola and Acari) are ambiguous with no evidence of the hypothesized pattern of attenuating effects. In fact, all significative responses of both Acari and Collembola are positive. This would seem, contrary to our hypothesis, to indicate a synergetic combined effect of KRE addition (mostly at intermediate and high concentrations). In all cases of significant response to a combined effect one or both components' response was neutral (nutrient addition especially). Contrasting and inverse results from nutrient and secondary metabolites addition have also been found for nematodes at intermediate KRE concentration with a negative ASC addition effect, positive nutrient addition effect and neutral combined effect. Direct nematocidal effects of plant secondary compounds have been documented in some cases, mostly in laboratory studies (Chitwood, 2002). If there was such a direct effect of knotweed we would expect it to also be present at the highest concentration, which is not the case in our study (Fig. 2).

These results provide the basis for further research on knotweed such as more detailed characterization of knotweed ASC and their potential allelopathic effects as well as further field work. As assessment of indirect, and direct, allelopathic effects of phenolic compounds on soil fauna has rarely been done. Thus, this report should provide useful data for authors working on such a subject as information is currently scarce on the subject. Finally, we hope the results presented here will provide useful reference data for future biological invasions study and inform managers of invaded areas on knotweed potential impacts.

5. Conclusion

In conclusion, the results showed an effect of knotweed rhizome extract (KRE) on soil microbiology. Fungal biomass remained unaffected but microbial biomass as a whole responded negatively to KRE in some cases. Interestingly these negative responses, when they occurred, were mostly attributable to factors other than the allelopathic secondary compounds (ASC) within KRE, most notably nutrient addition. Calculated responses of microbial biomass to ASC addition were, albeit unsignificantly so, positive. While KRE addition had an effect in most cases on taxa “higher” within the soil trophic networks, there were no evident and generalizable trophic cascades across trophic levels for a given KRE concentration. Path analysis did reveal important changes in soil food web structure (constructed based on hypothesized producer-consumer relationships) which appeared to be mostly within the bacterial pathway, and concentration-dependent. There was circumstantial, but not generalizable, evidence of compensating, or attenuating, effects of nutrient and ASC addition on various taxa. Rarely was ASC effect, when documented, concentration-dependent in the results.

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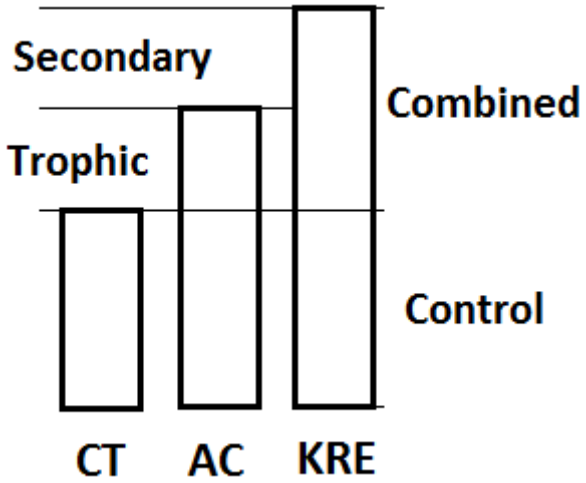
753 Figures



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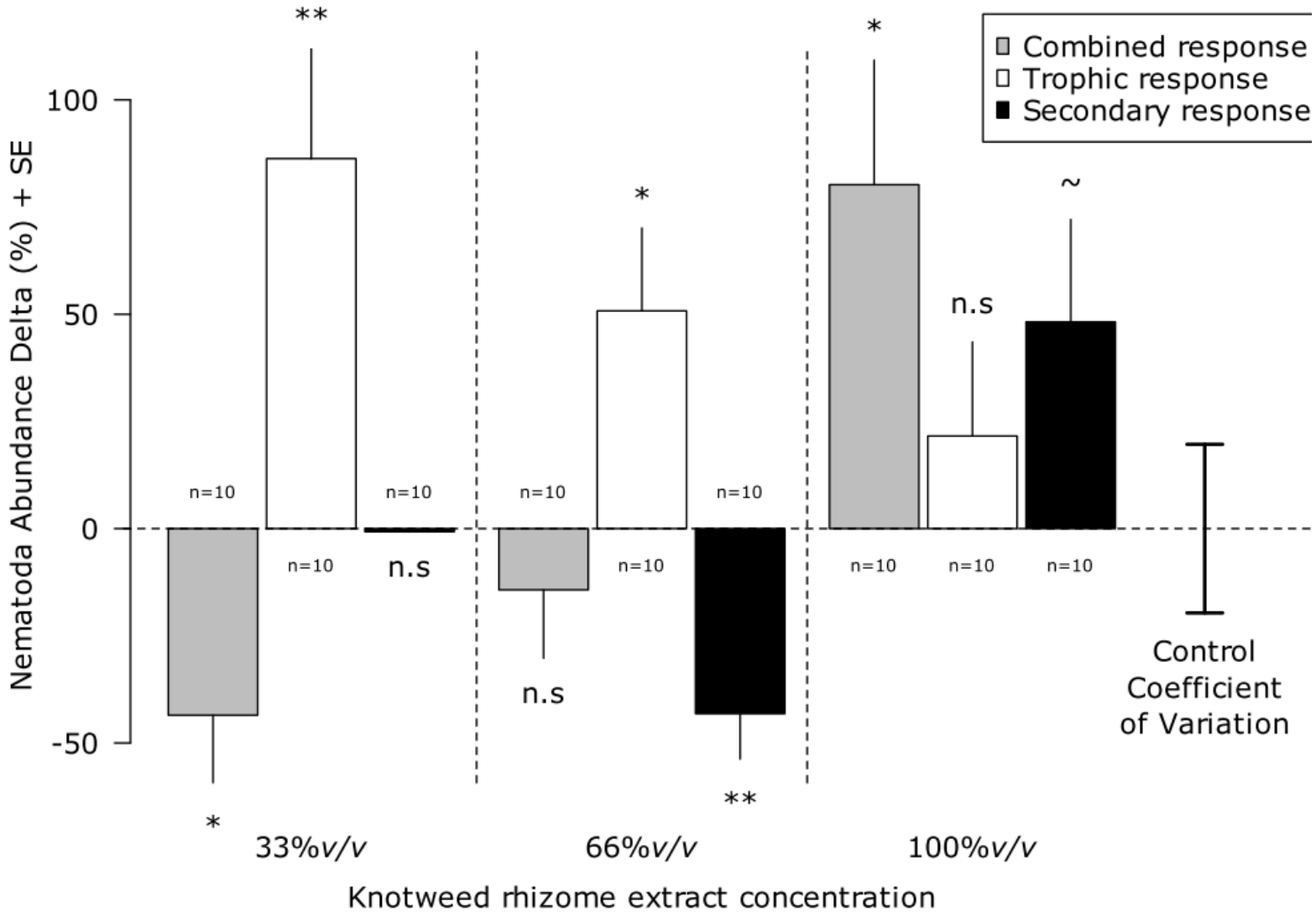
755 Figure 1 : Diagram of calculations involved in separating knotweed rhizome extract (KRE) effects. CT: control, AC: activated carbon filtration, KRE:

756 no AC filtration.


Combined response
Trophic response
Secondary response
Nematoda Abundance Delta (%) + SE
100
50
0
-50
**
*
*
*
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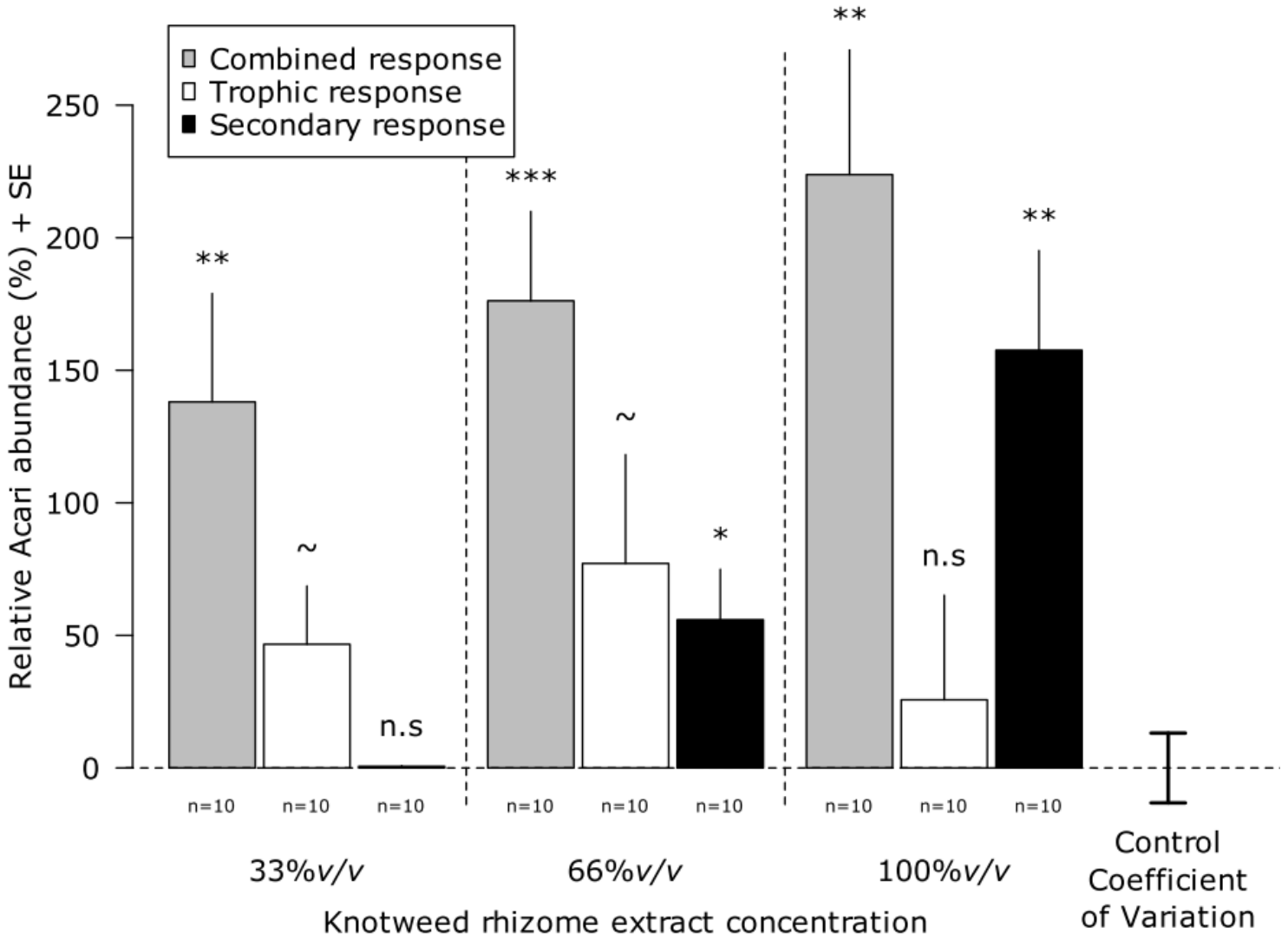
n.s
n.s
n.s
**
n=10
n=10
n=10
n=10
n=10
n=10
n=10
n=10
n=10
Control
Coefficient
of Variation
33%v/v
66%v/v
100%v/v
Knotweed rhizome extract concentration

757

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


Combined response
Trophic response
Secondary response
Relative Acari abundance (%) + SE
250
200
150
100
50
0
**
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n.s
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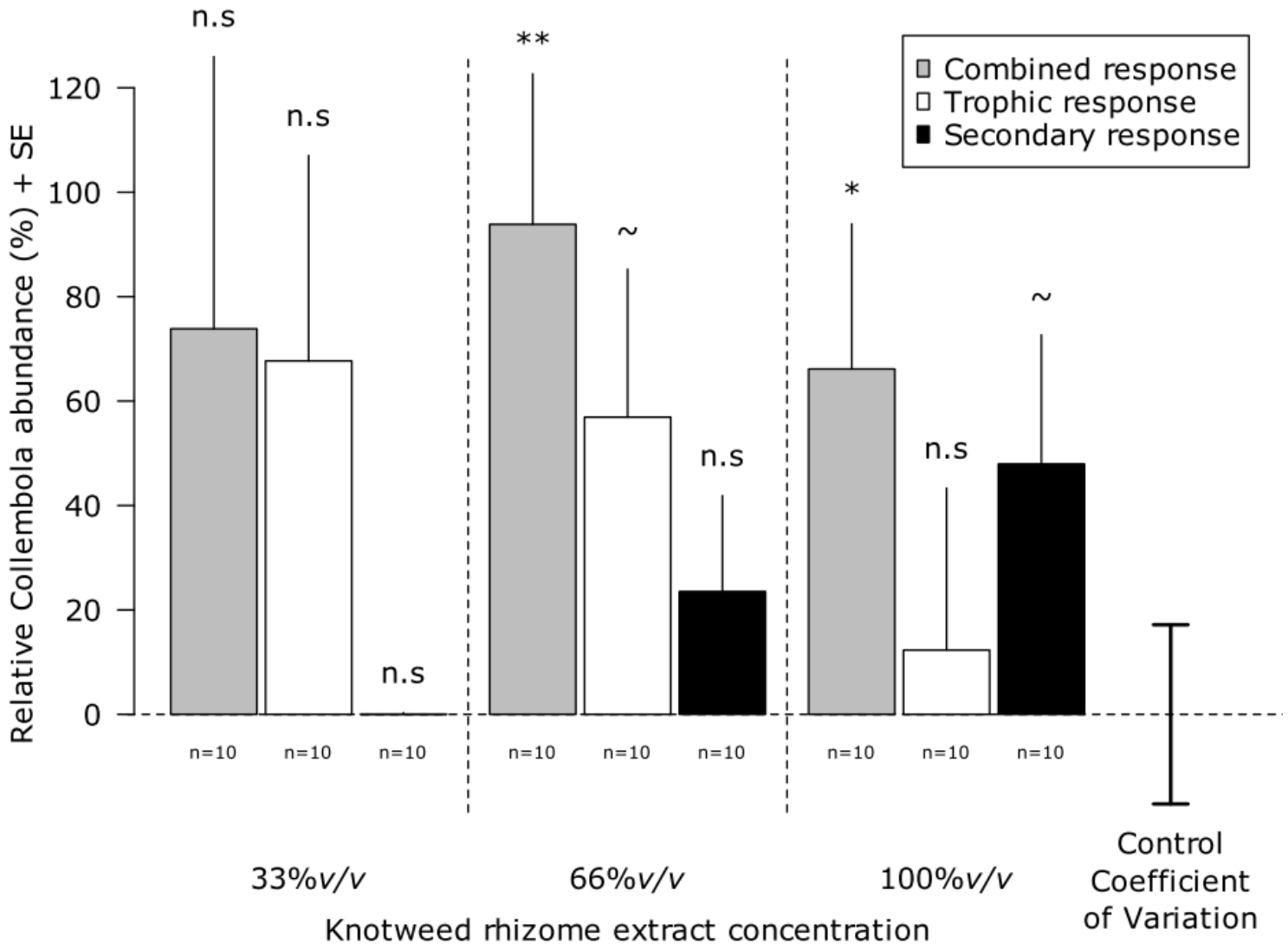
~
*
**
n.s
**
n=10
33%v/v
66%v/v
100%v/v
Control
Coefficient
of Variation
Knotweed rhizome extract concentration

761

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

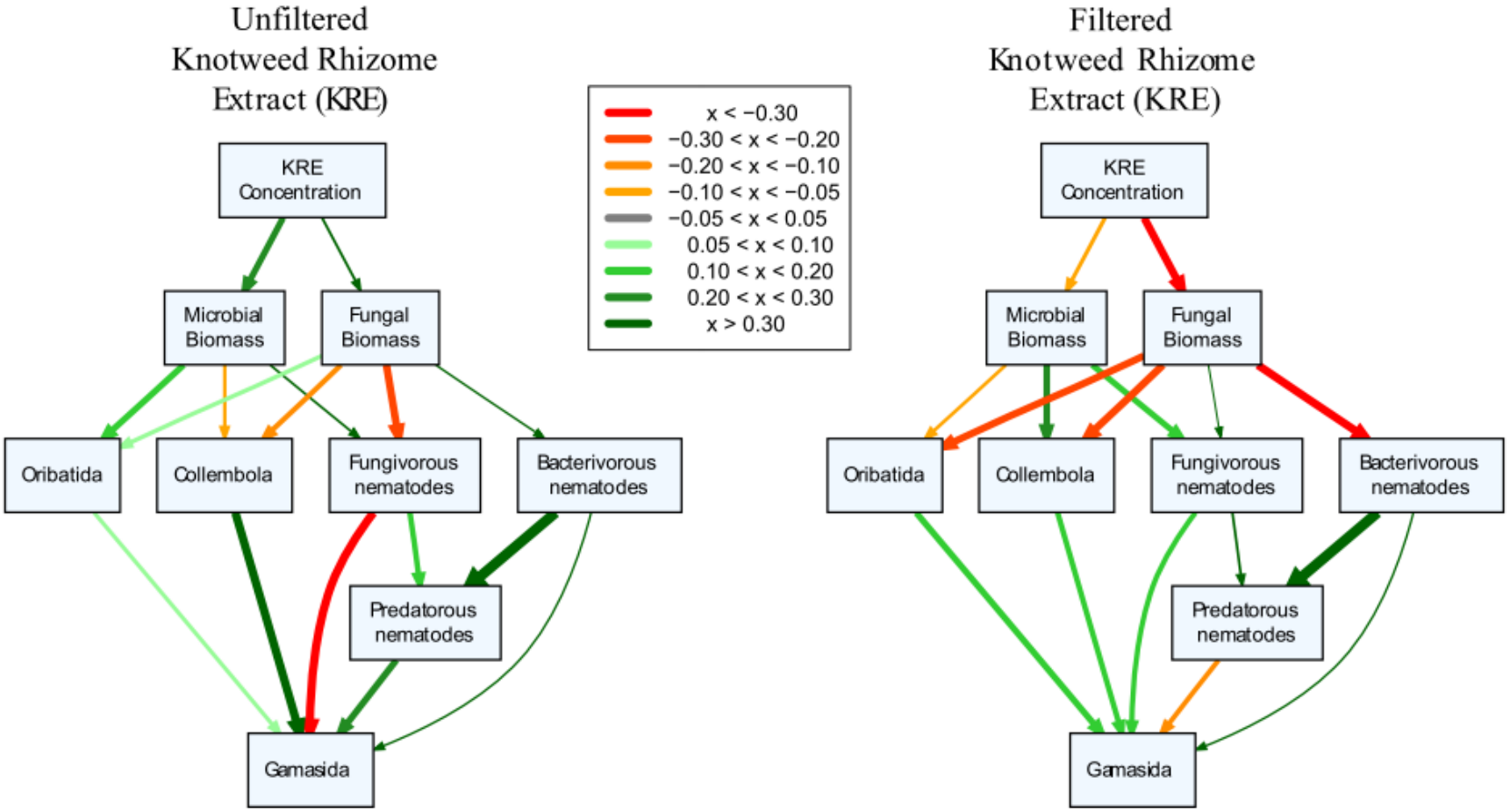
763 with decomposition of effects. Symbols indicate levels of significativity of repeated statistical testing of differences between calculated values and

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


n.s
n.s
n.s
**
~
n.s
*
n.s
~
Combined response
Trophic response
Secondary response
Relative Collembola abundance (%) + SE
120
100
80
60
40
20
0
n=10
n=10
n=10
n=10
n=10
n=10
n=10
n=10
n=10
33%v/v
66%v/v
100%v/v
Control
Coefficient
of Variation
Knotweed rhizome extract concentration

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



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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.

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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	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.
	-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
pH (KCl)	$p < 0.10$	$p < 0.01$	$p < 0.05$	$p < 0.10$	n.s.	n.s.	n.s.	$p < 0.05$	$p < 0.05$
	-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
C/N ratio	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
Shannon's diversity	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
	p < 0.05	n.s.	n.s.	p < 0.001	n.s.	p < 0.05	n.s.	p < 0.01	p < 0.10
Functional richness	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
	n.s.	n.s.	n.s.	p < 0.05	n.s.	p < 0.05	n.s.	n.s.	p < 0.10
Functional evenness	-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
	n.s.	n.s.	n.s.	p < 0.05	n.s.	p < 0.05	n.s.	n.s.	n.s.