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Impact of *Artemisia absinthium* hydrolate extracts with nematicidal activity on non-target soil organisms of different trophic levels

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ABSTRACT

Natural pesticides are considered a good alternative to synthetic pesticides to reduce environmental impacts. However, biopesticides may have unknown effects on the environment, and can affect non-target organisms. In this study, the ecotoxicological effects of an aqueous extract (hydrolate) from Spanish populations of *Artemisia absinthium* (var. Candial) showing a promising biopesticide activity, were evaluated on non-target soil organisms from different trophic levels (natural microbial communities characterized through 16S rRNA gene sequencing, the earthworm *Eisenia fetida* and the plant *Allium cepa*). The hydrolate usually was considered as a by-product of the distillation to obtain essential oils. However, recently has been found to have nematicide properties. The hydrolate caused acute toxicity at values of LC_{50} of 3.87% v/v for *A. cepa* and 0.07 mL/g for *E. fetida*. All the concentrations except for the most diluted (1% v/v) reduced the bacterial physiological activity compared to controls (LC_{50} = 25.72% v/v after 24 h of exposure). The hydrolate also slightly altered the ability of the microbial community to degrade carbon substrates. These results indicate that the hydrolate from *A. absinthium* may affect the survival and metabolic abilities of key soil organisms.

1. Introduction

The widespread application of synthetic pesticides has become a continous practice during the last 100 years due to the need to obtain enough food for an exponentially growing population (Liu et al., 2015). That intensive application provoked the accumulation of a wide variety of synthetic pesticides in soils, and terrestrial and aquatic ecosystems (Carvalho, 2017; Kelepertzis, 2014; Tejada et al., 2017). The continuous exposure to pesticides damaged non-target organisms, affected biodiversity and led to the generation of resistant varieties of pests among other consequences (Carvalho, 2017; Komarek et al., 2010; Rosner and Markowitz, 2013; Shao and Zhang, 2017). These problems are also associated with the use of synthetic nematicides and the generation of their degradation products, provoking undesired environmental impacts and side effects on human health (Haydock et al., 2006; Sanchez-Moreno et al., 2010).

Plant-based pesticides are increasingly used to replace synthetic pesticides in pest control. For example, different plant-based nematicides (bionematicides) are extracted from agricultural residues and by-products as roots, fruit skins, flowers, seeds, bark, leaves and, stems (Timper, 2014). These products derived from plants with a pesticidal action seem to have advantages that make them less harmful to the environment than synthetic ones (Benelli, 2015; Govindarajan and Benelli, 2016; Govindarajan et al., 2016; Pavela, 2015). These advantages allowed many plant-based formulations to be described as "Generally Recognised as Safe" (GRAS) by the Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) of the USA (Kedia et al., 2015).

Usually synthetic pesticides are based on one or a few ingredients, but formulations from biopesticides are a mixture of compounds. This could reduce the risk of pests developing resistance to the pesticide (George et al., 2014; Jaya et al., 2014; Miresmailli et al., 2006). Other advantage of plant-derived pesticides is that their active substances could be more easily degraded by environmental factors as temperature, solar radiation or enzymatic activity (El-Wakeil, 2013; Varma and Dubey, 1999).

Many plant-derived products exerting a pesticidal action have been obtained from the Artemisia genus (Duke et al., 1988).

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Artemisia absinthium L (wormwood) is one of the most widely distributed species of the Artemisia genus. This plant has abundant oil-producing glands that provide its intense aromatic properties (Chiasson et al., 2001; Mihajilov-Krstev et al., 2014).

A new variety of Artemisia absinthium (var. Candial) was obtained from domesticated Spanish populations that showed a chemical stable composition (Bailen et al., 2013; Gonzalez-Coloma et al., 2012; Julio et al., 2015; Martin et al., 2011). When steam distillation (or hydrodistillation) is carried out to obtain essential oils from a plant material, the volatile compounds are extracted together with the water in the vapor stream flows. In the condensation, the non-soluble fraction (the essential oils) appears on the surface of the aqueous phase and can be separated by decantation. What remains is the hydrolate, the mixture of water and soluble compounds, until now considered a mere by-product. The hydrolate of this domesticated *Artemisia* populations has been described (Julio et al., 2017a); it contains different water-soluble volatile compounds and large amounts of active ingredients such as acids, aldehydes and amines. The properties of the hydrolate as biopesticides of *A. absinthium* were studied and have been quite promising. For example, this hydrolate presented trypanocidal and leishmanicidal activities (Gonzalez-Coloma et al., 2012; Martinez-Diaz et al., 2015) as well as antifungal (Julio et al., 2015), and strong nematocidal properties (Bailen et al., 2013; Garcia-Rodriguez et al., 2015; Julio et al., 2017b). Other aqueous extracts of Artemisia absinthium also showed fungicidal properties (Ali et al., 2015).

Even if a large number of studies have focused on the biological activity of plant-based products with a pesticidal action on target organisms (Alarcon and Cespedes, 2015; Carlsen and Fomsgaard, 2008; Di Fabio et al., 2014; Yoon et al., 2013), studies concerning toxicological studies and their effects on non-target organisms are scarce (Chelinho et al., 2017; Shao and Zhang, 2017; Singh et al., 2015). The recent developments on the use of natural nematicides—specifically bionematicides—raises concern about their impact on the natural soil biota (Ntalli and Caboni, 2012).

Due to the almost unlimited resource of bioactive plant products, the great progress in their isolation and characterization, as well as new European legislation that encourages the development of less harmful substances (Villaverde et al., 2016), the use of biopesticides will grow in the near future. To proceed with the registration and commercialization of the obtained products, they will need the same state and regulations as synthetic pesticides. Therefore, the potential risks of soil application on non-target organisms should be evaluated and include different trophic levels.

This study is the first attempt to characterize the soil ecotoxicity of the *A. Absinthium L* hydrolate using key model soil organisms from different trophic levels: a plant (assessing the effects on root growth of *Allium cepa L.*), a soil invertebrate (assessing the lethality on *Eisenia fetida*) and the soil microbial community (assessing the ability to degrade different carbon sources). In addition to those physiological endpoints, a taxonomic analysis of the microbial community was done which will eventually allow for a deeper understanding of the hydrolate effects on these key elements of soil ecosystems.

2. Material and methods

2.1. Extraction of the vegetal material

A population of domesticated Artemisia absinthium (var. Candial) was harvested in an experimental cropland (Ejea de los Caballeros, Zaragoza, Spain) in the summer of 2016 (Julio et al., 2017b). The hydrolate was produced in an experimental research plant belonging to the Aragon Regional Government (http://www.cita-aragon.es) by steam-distillation for 1 h (Julio et al., 2017b).

2.2. Ecotoxicity assay with E. fetida

The toxicity tests were carried out following the indications of the OECD 207 (1984) methodology in a similar way to that described before (Pino et al., 2015). In summary, adult individuals of *Eisenia fetida were acquired from the composters of TODOVERDE* (Galicia, Spain).

All the earthworms selected were adults (above 60 days of age) with clitellum and similar size (weights ranging from 300 to 600 mg). Before testing, earthworms were acclimatized over 15 days in a sphagnum peat conditioned substrate from the Spanish FLOWER company (Tarragona, Spain). They were kept at a stable condition: 18–25°C, pH 7.5–8 and 80–85% humidity.

The toxicity tests were carried out in polypropylene containers of 1 L capacity with lid. Small holes were made in the lid that allowed ventilation but reduced moisture loss.

The soil for the tests was prepared with the following mixture, according to the OECD 207: industrial fine sand (Imerys Ceramics España, S.A., Spain), sphagnum peat (Verdecora vivarium, Spain), and kaolin clay (Imerys Ceramics España, S.A., Spain) in a 7:1:2 ratio. 500 g was poured in each of the test containers. The moisture of the substrate was adjusted with deionized water in an amount equivalent to 35% of the dry weight of soil and pH was adjusted to 6.0 ± 0.5 by addition of calcium carbonate.

Finally, 10 adult earthworms were added to each testing jar (x 3 replicates). Hydrolate was diluted in distilled water at the following dilutions: 0.005-0.01-0.05-0.1-0.3 (mL/mg). Negative controls were prepared using the same procedure without the hydrolate.

The containers were kept under controlled environmental conditions: 20 ± 2°C, 80–85% relative humidity and 400–800 lx of light. Mortality of earthworms was measured after 14 days of treatment.The LC50 values were calculated using log-Probit analysis (Bliss, 1934).

2.3. Allium cepa assay

Acute toxicity experiments with *A. cepa* were conducted according to Fiskesjö, 1993). Bulbs of *A. cepa* (var Stuttgarter Riesen de 14/21) were acquired from Fitoagrícola Company (Spain). Young onion bulbs were placed in 15 mL tubes using mineral water (VERI, Aguas de San Martín de Veri S.A., Spain) as the growth medium because of its adequate content in Ca + Mg (https://www.veri.es/es/el-producto). Ecotoxicological tests were performed using 6 replicates for each concentration: negative control-0.1-1-10-50 (% v/v) and incubating the bulbs in a dark chamber at 20°C over 72 h. The test solutions were renewed every 24 h.

2.4. Biolog EcoPlate™tests

2.4.1. Soil samples

The soil was obtained from an experimental crop field free of pesticides or other contaminants (CSIC, Montañana, Zaragoza, NE-Spain). The samples were collected on October 30, 2017 and taken to the laboratory in less than 15 min.

The soil was sieved at < 2 mm and stored in polypropilene jars with 2 L volumes at 4°C until use. The texture of the soil was: 37.3% sand; 24.7% silt and 38.0% clay; the content of organic matter was 3.86% and the pH was between 7.6 and 8.

2.4.2. Genetic study of soil microorganisms

Soil microbes were extracted by mixing 10 g of soil with 95 mL of sterilized Milli-Q water in 100 mL Erlenmeyer flasks; this method has been developed in previous studies (Muniz et al., 2014; Pino-Otin et al., 2017; Tiquia, 2010). The soil suspension was magnetically stirred for 30 min, followed by 1 h of rest. Afterward, 10 mL of the soil suspension was transferred into 50 mL Falcon tubes and—after 1 min of sonication—the tubes were centrifuged (1000 g × 10 min). Then, 9.5 mL of the supernatant were separated and the remaining was resuspended by

adding 9.5 mL of water. After repeating the above protocol five times, 47.5 mL of soil lixiviate were obtained from each sample. The lixiviates were finally centrifuged at 5000 g and stored at −80°C.

Genetic sequencing was done as described in (Pino-Otin et al., 2019) at the at Genomics Unit Cantoblanco, Science Park, Madrid (Spain). Briefly, bacterial DNA was digested using RNAse and proteinase K enzymes and extracted using G-spin™ columns (INTRON Biotechnology). Amplification of the V3–V4 region of the 16 S rRNA gene was carried out according to the technique of amplicon sequencing (Caporaso et al., 2012; Caporaso et al., 2011). Bioanalyzer 2100 (Agilent) was used to analyse Individual amplicon libraries. An Illumina MiSeq Instrument served to sequencing DNA samples under a 2x300 protocol. Finally, metagenomics 16S, were filtered qualitatively and mapped (see Pino-Otin et al., 2019 for more details).

A high percentage of taxa within each level of organization was successfully sequenced (above 90%) except at the genus (82.79%) and species (26.36%) levels (Support information SI2). The relative abundance of the highest 8 taxonomic classifications at each level are shown in Fig. 3. The complete taxonomic classification is provided in Support information SI1. The sequencing is based on 209.275 total reads whose 94.0% passed the quality filtering.

2.4.3. Sample exposition to hydrolate and Biolog EcoPlate™ assay

The metabolic performance of microbial communities was assessed as the ability to degrade different carbon sources after hydrolate exposure, using Biolog EcoPlate™. This approach is widely used for characterizing the metabolic abilities –substrate preference-of microbial communities (Yu et al., 2012). That method allows for comparing the toxicity of different compounds on microbial communities (Muniz et al., 2014; Pino-Otin et al., 2017; Tiquia, 2010). Biolog EcoPlates presents wells with 31 different carbon sources and tetrazolium violet, whose reduction indicates the ability of microbes to degrade every carbon sorce (Pohland, 2009).

Soil lixiviate (47.5 mL) containing microbes was extracted and conserved as detailed in section 2.4.2. Mineral soil particles were removed by centrifugation (500g × 2 min). A volume of 150 µL of dilutions of *A. absinthium L* hydrolate (100%-50%-25% −10% and 1% v/v in distilled water) were deposited in the wells of the Biolog plates (1 per concentration), and 150 µL of the lixiviate containing the soil microorganisms were added. All manipulations were performed under conditions of sterility in a flow chamber. Plates were incubated 7 days at 25°C in darkness and sterile conditions. The final dilution pH ranged between 7.71 and 8.06. The optical density (OD at 590 nm) of all Biolog plate wells was measured at time 0 (just after the incubation) and every 24 h using a Microplate Reader (model Anthos, 2010) and the data was acquired and processed using ADAP 2.0 software (Biochrom, Ltd. Cambridge Science Park, Cambridge, England).

2.5. Statistics and graphical representation

Experimental data for *E. fetida* and *A. cepa* was adjusted to dose-response curves using XLSTAT Soft. (2014.5.03). Thus, the EC_{50} (effect concentration) or LC_{50} (lethal concentration) values and standard errors (SE) were calculated. Chi-square test was used to evaluate the significance of the models.

Results from Biolog EcoPlate™ assays, was represented as the average well colour development -AWCD- (see detailed method in Muniz et al., 2014) as follows:

$$AWCD = \sum_{i=0}^{i=12} \left(OD_{t=x_i} - OD_{t=x_0}\right) \quad (1)$$

where OD_i is the well optical density at time *i*,and $OD_{t\ =\ x0}$ that at time 0. Values of ODs at AWCD plateau were used to calculate the physiological diversity, using Paleontological Statistics Software v. 3.0 (Fang et al., 2009; Pino-Otin et al., 2017; Tortella et al., 2013) as follows:

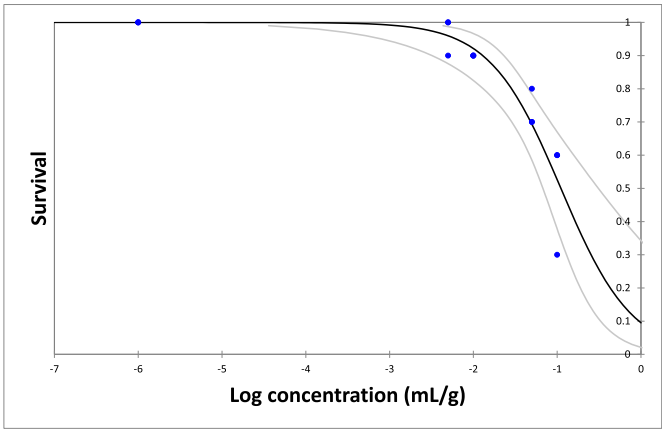

Fig. 1. The black curve shows the dose-response of *Eisenia fetida* after exposure to *Artemisia absinthium L* hydrolate during 14 days and represents the average value of three replicates. Grey lines are the confidence limits (95%).

- Shannon-Weaver index (as a measure of richness):

$$H = -\sum . p \log_2 pi \quad (2)$$

where p_i is the ratio of absorbance of a particular well to the sum of absorbances of all microplate wells. The Shannon index, as metabolic diversity proxy, was computed considering each carbon source as a different species, and its color intensity, as its abundance. Student's t-test were used to compare samples, using XLSTAT Soft. (2014.5.03).

3. Results

3.1. Mortality of A. absinthium L hydrolate on E. fetida

Dose-response curve of *E. fetida* after 14 days of exposure to *A. absinthium L hydrolate is shown in* Fig. 1. Significance was evaluated with the chi-square test and all values were very significant ($P < 0.0001$). The measure effect is the mortality of the earthworm.

As can be seen, the hydrolate cause a great impact on the survival of *E.fetida* with an LC_{10} = 0.02 mL/g (s.e. interval of 0.008–0.03) and an LC_{50} value of 0.07 mL/g (s.e. interval of 0.05–0.10) of hydrolate dilution.

3.2. Effects of A. absinthium L hydrolate on A. cepa

After 72 h of exposure, the hydrolate strongly inhibited the growth of the roots, yielding an LC_{10} value of 0.18% v/v (s.e. interval of 0.13–0.23%) and an LC_{50} value of 3.87% v/v (s.e. interval of 3.30–4.53%) of the dilution (see Fig. 2). All values were highly significant ($P < 0.0001$).

3.3. Impact on diversity and physiology of soil bacteria

3.3.1. Genetic analysis of microbial populations

The highest 8 taxonomic classifications at each level and their relative abundance can be seen in Fig. 3. All taxa found in the samples as well as their abundance can be consulted in Support information SI1.

Sequencing comes from 209,275 total reads and 196,682 reads passed filter of quality (94.0%).

Each level of organization was successfully sequenced (above 90% of taxa). A little less in the case of genus and species (96.91% and 41.00%, respectively) (Support information SI2).

The charts in Fig. 4 show the % of taxa abundance for different taxonomic levels. The predominant phylum (Fig. 3) was Proteobacteria (76.06%), then Bacteroidetes (11.29%) and Firmicutes (4.86%). Proteobacteria were the dominant phylum of non-contaminated soils

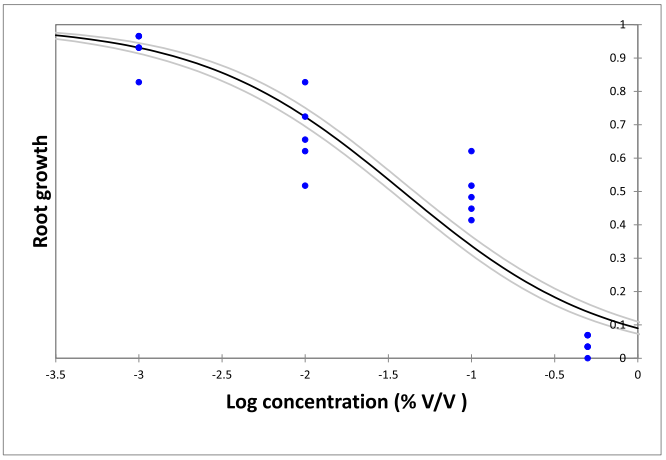

Fig. 2. The curve shows the dose-response of *Allium cepa* after exposure to *A. absinthium L* hydrolate during 72 h. As before, grey lines are the confidence limits (95%). Curve is the average value of six replicates. The measured effect is the inhibition of the growth of the roots.

(Madigan et al. (2015); (Janssen, 2006; Spain et al., 2009). Bacteroidetes were also abundant groups; Firmicutes somewhat less so (Madigan et al., 2015). However, in our samples, they appear practically in the same proportion.

The diversity of Bacteroidetes seems to be a frequent feature in uncontaminated soils (Madigan et al., 2015). It is also common to find a significant proportion of unclassified microorganisms' phylotypes or minority bacterial members (Madigan et al., 2015) due to the high typical bacterial diversity of edaphic ecosystems (Spain et al., 2009).

Among Proteobacteria, the most abundant were the Gammabacteria class (39.21% of Proteobacteria, 29.83% of the total taxa), followed by Beta (33.62% of proteobacteria, 25.57% of the total taxa) and Alphaproteobacteria class (18.07% of Proteobacteria, 13.75% of the total taxa). Although Alphaproteobacteria is usually the most frequent family of soil Proteobacteria (Janssen, 2006; Spain et al., 2009), the proportions between these three families may vary depending on the characteristics of the soil (Faoro et al., 2010). Pseudomonadales order was the most abundant among the Gammaproteobacteria class (91.27% of Gammaproteobacterias; 27.22% of the total taxa), and all of them were of the Pseudomonas genus.

Betaproteobacteria have a wide range of ecological and metabolic characteristics (Madigan et al., 2015). Almost all the Betaprotebacteria identified were of the order Burkholderiales (91.92% of Betaproteobacteria and 23.50% of total taxa). This order is also frequently comprised of taxa of the soil and water that intervene in the carbon and nitrogen cycles (Madigan et al., 2015). Among Burkholderiales, the most abundant family was Oxalabateriaceae (81.55% of Burkholderiales and 19.17% of the total) followed by the Comamonadaceae family (17.79% of Burkholderiales and 4.18% of total taxa). The main genus of Oxalabateriaceae was Janthinobacterium (86.85% of this family and 16.65% of the total).

Members from the Oxalobacteraceae family are commonly found in the soil and rhizosphere (Aleklett et al., 2015; Green et al., 2007), including the genus Janthinobacterium (Scheublin et al., 2010). The high presence of these groups in our samples could be due to the inclusion of epiphytic members of the root microbiota, which is where bacteria associated with water films and soil particles of the root surface would be expected.

The Alphaproteobacteria class showed greater diversity than the Gamma and Betaproteobacterias. This class, with almost 1000 species described, includes most of the oligotrophic bacteria capable of living in an environment that offers very low levels of nutrients.

The order Sphingomonadales in the sample (41.32% of the Alphaproteobacteria of the sample, 5.68% of the total taxa) was the

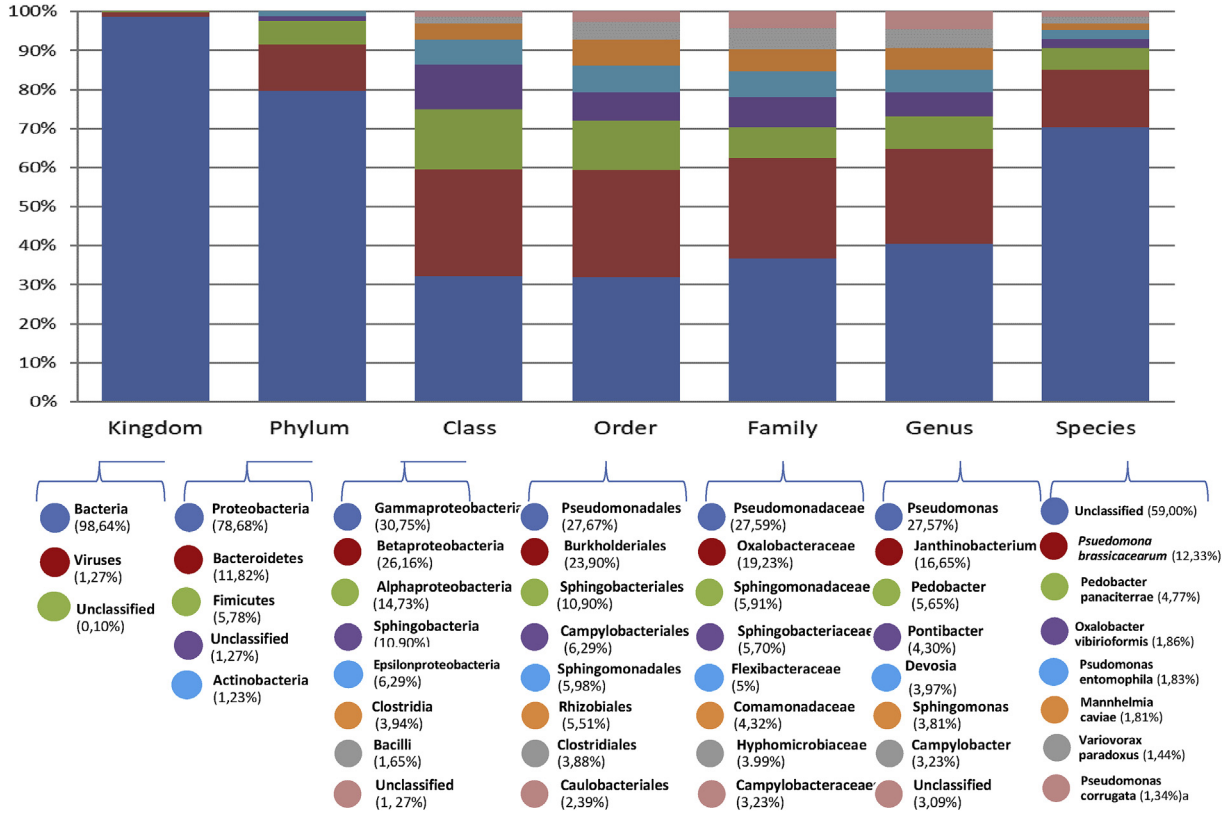

Fig. 3. Eight more abundant taxa in each taxonomic level found in the samples obtained from an experimental field (Zaragoza, NE Spain). The percentages indicate relative abundances within each level.

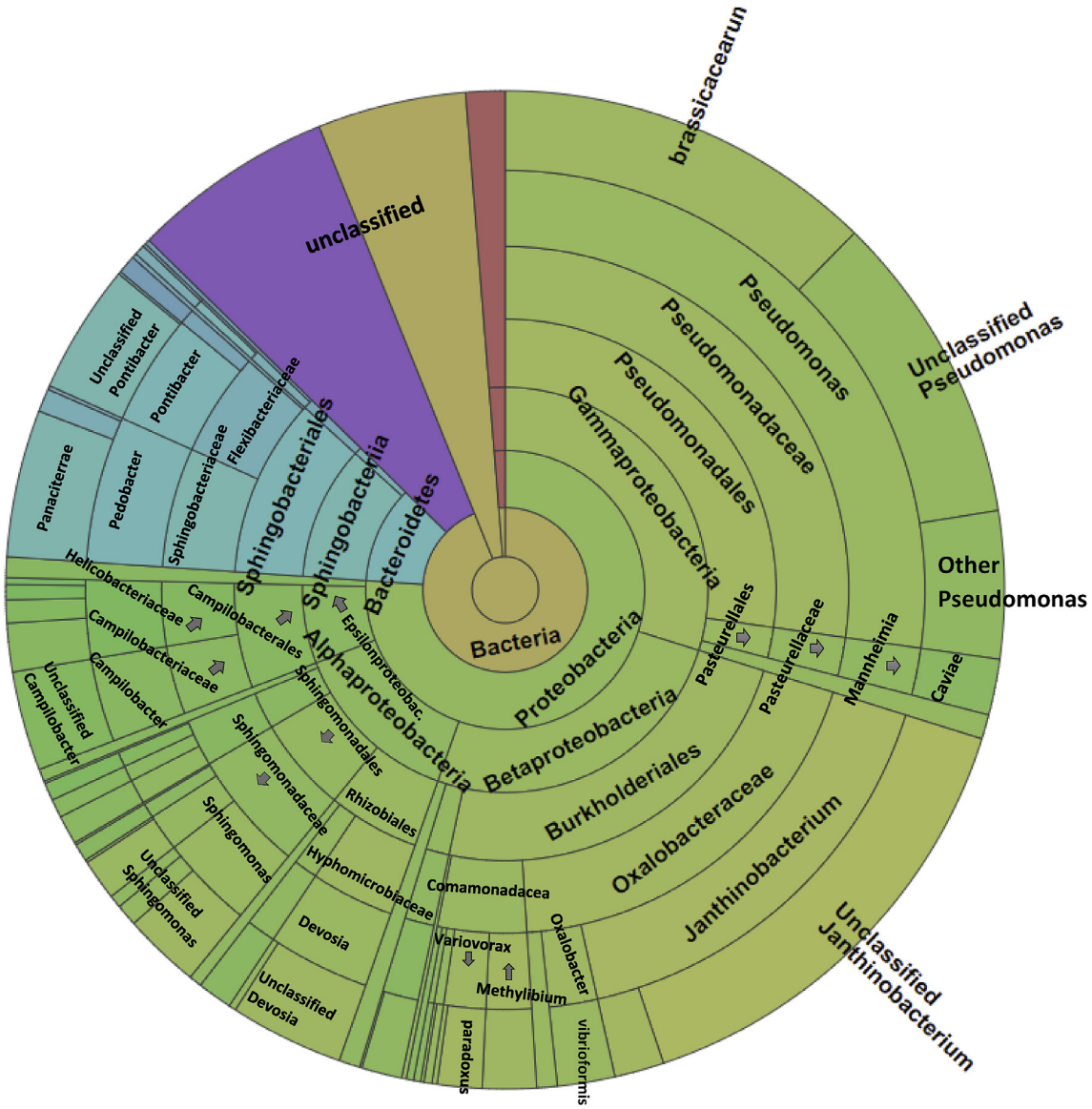

Fig. 4. The graph shows the abundance of taxa for different taxonomic levels found in the samples obtained from an experimental field (Zaragoza, NE Spain). From outside the circle to inside: species, genus, family, order, class and phylum.

most abundant, following by the order Rhizobiales (39.78% of Alphaproteobacteria; 5.47% of the total taxa) and the order Caulobacterales (15.98% of this class; 2.20% of the total). Sphingomonas (63.62% of the Sphingomonadaceae family; 3.61% of the total) and Devosia (100% of the Hyphomicrobiaceae family and 3.92% of the total) were the genera most abundant among the order Rhizobiales.

Sphingomonas strains have been isolated from a great variety of soil environments (Ko et al., 2017; Liu et al., 2016)—including rhizosphere soil (Daane et al., 2001)—and they are versatile in their nutrition and able to metabolize an extensive range of organic compounds (Madigan et al., 2015). In addition, Sphingomonas have shown unique abilities to degrade a variety of pollutants, including insecticides (Nagata et al., 1999) and herbicides (Adkins, 1999). Devosia are common bacteria from the rhizosphere (Nor et al., 2017).

3.3.2. Average well colour development (AWCD)

Fig. 5 shows the average well colour development (AWCD) of the BIOLOG plate plotted for 7 days. Points are the average of three replicates. All the hydrolate concentrations—except for the most diluted (1% v/v)—decreased the bacterial metabolism measured as AWCD compared to the control. A concentration of 10% hydrolate leads to a small decrease in the values of AWCD after 96 h. This decrease becomes significative (Student's t-test, $P \leq 0.05$) at the 25–50 and 100% concentrations (v/v). However, the smallest concentration (1%) caused a stimulating effect on metabolism. The AWCD data at 24 h of exposure was also represented as a dose-response curve (see Support information SI3). That allowed for the calculation of an EC_{10} value of 13.46% v/v (10.99–15.57) and an EC_{50} value of 25.72% V/V (23.29–28.24). These toxicity values were highly significant (Chi-square test, $P < 0.0001$).

3.3.3. Physiological diversity of soil microorganisms communities

Physiological diversity of soil bacterial communities was calculated from the ODs values at the plateau of the AWCD. After *A. absinthium* L hydrolates exposition, none of the doses produced significant differences in the physiological diversity of soil bacteria (Fig. 6) compared to the control (Student's t-test, $P \leq 0.5$).

3.3.4. Physiological diversity of soil microorganisms substrate utilization

In order to highlight general patterns of substrate utilization, all

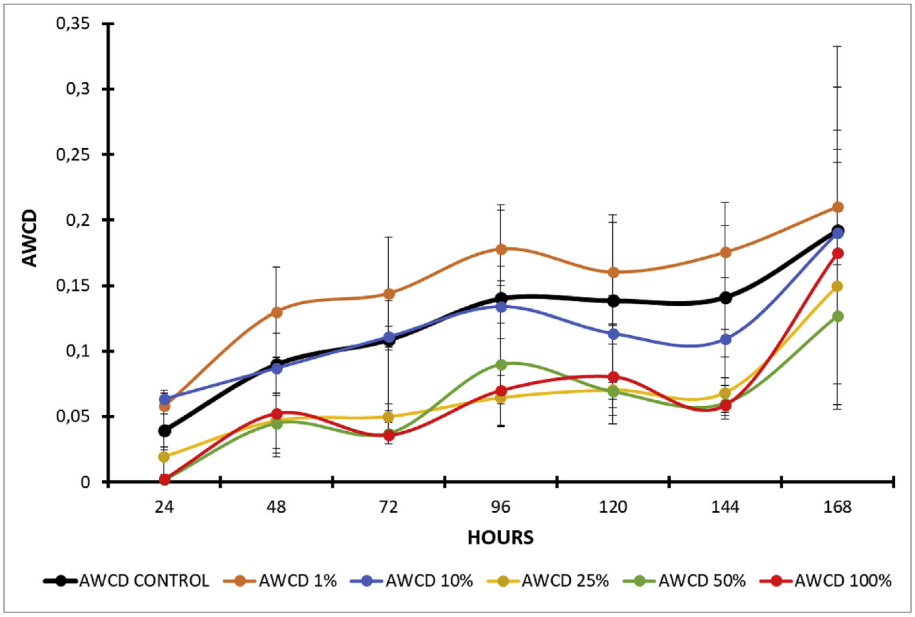

Figure 5. 168-h incubation of soil microorganisms exposed to *A. absinthium* L hydrolate concentrations (% v/v). Curves are the Average Well Color Development (AWCD) in Biolog EcoPlates of metabolized substrates. Points in curves are the average value of three replicates. Black curve is the control (bacteria only treated with water). The standard deviation of mean of the three replicates are represented by error bars.

carbon sources were evaluated grouped into 5 groups as described before (Lehman et al., 1995; Pino-Otin et al., 2019; Pino-Otin et al., 2017; Weber and Legge, 2009; Zak et al., 1994). The exposure to hydrolate increased the capacity to metabolize almost all substrate classes compared to the control (Fig. 7), with the exception of amines/amides that generally tended to decrease. The highest concentration of hydrolate (100%) provoked an increase in the metabolism of carbohydrates ($P = 0.03$), carboxylic and ketonic acids ($P = 0.002$) and the reduction of the metabolism of amino acids ($P = 0.005$). H-Shannon values of carboxylic and ketonic acids also increased significantly at a concentration of 50% ($P < 0.05$); carbohydrates and amino acids also increased ($P > 0.05$) to that of 25%. The exposure to hydrolate concentrations of 10% and 1% resulted in similar metabolic profiles.

4. Discussion

4.1. Effects on non-target organisms A. cepa and E. fetida

The hydrolate presented intense phytotoxicity in the case of the root grown by A. cepa and in the survival of the earthworm E. fetida.

The monoterpene (−) - (Z)-2,6-dimethylocta-5,7-diene-2,3-diol was found to be the major active component of the hydrolate of A. absinthium. This compound is responsible for the biopesticide activity of the hydrolate (Julio et al., 2017b).

This monoterpene has a low molecular weight of 170.25 g/mol (Wishart et al., 2018), water solubility of 715 mg/L, pka = 20.5 and log P = 3.1 (Pino-Otin et al., 2019). It is weakly acidic and will ionise with difficulty. All these characteristics will increase its ability to cross biological membranes and to affect biological systems. Accordingly, other monoterpenoids—including some acyclic alcohols like farnesol, citronellol or geraniol—have also been reported as bionematicides against a model nematode: Caenorhabditis elegans (Abdel-Rahman et al., 2013). However, we must not lose sight of the fact that this compound, despite being the majority, is in a mixture with many others that could modify these physicochemical characteristics.

Even if bionematicides are considered safer alternatives to synthetic ones, few studies have demonstrated that these products may also affect non-target soil organisms (Chelinho et al., 2017). These products (in this case the active molecule was naphthoquinone) affected seed germination of *Zea mays* and *Brassica napus* as well as another earthworm (*E. andrei*).

The inhibition of root growth during the *A. cepa* tests may rely on the effects at root cells. Other monoterpenes (1,8-Cineole) produced by *Sarracenia leucophylla* inhibited root growth and DNA synthesis in the

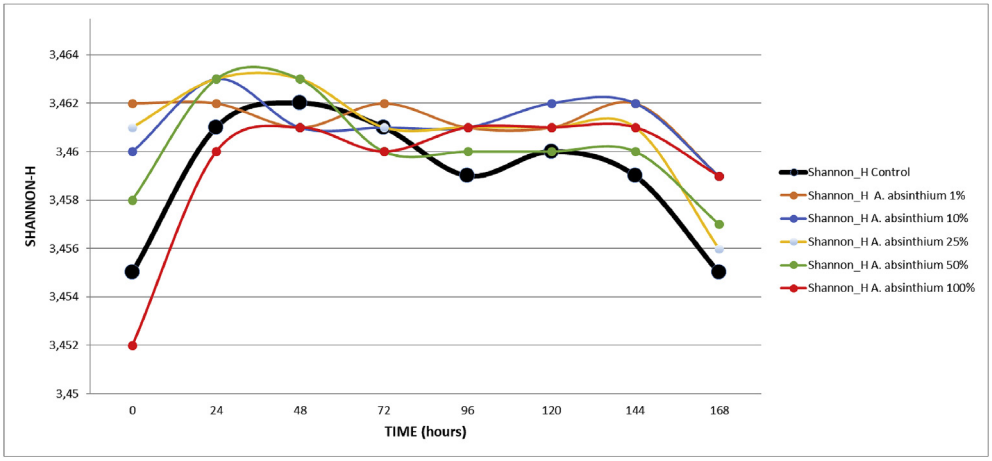

Fig. 6. Curves represent the variation of the Shannon–Weaver index of soil bacteria communities treated with *A. absinthium* L hydrolate during 168 h. Concentrations are in % V/V. Black line are the control.

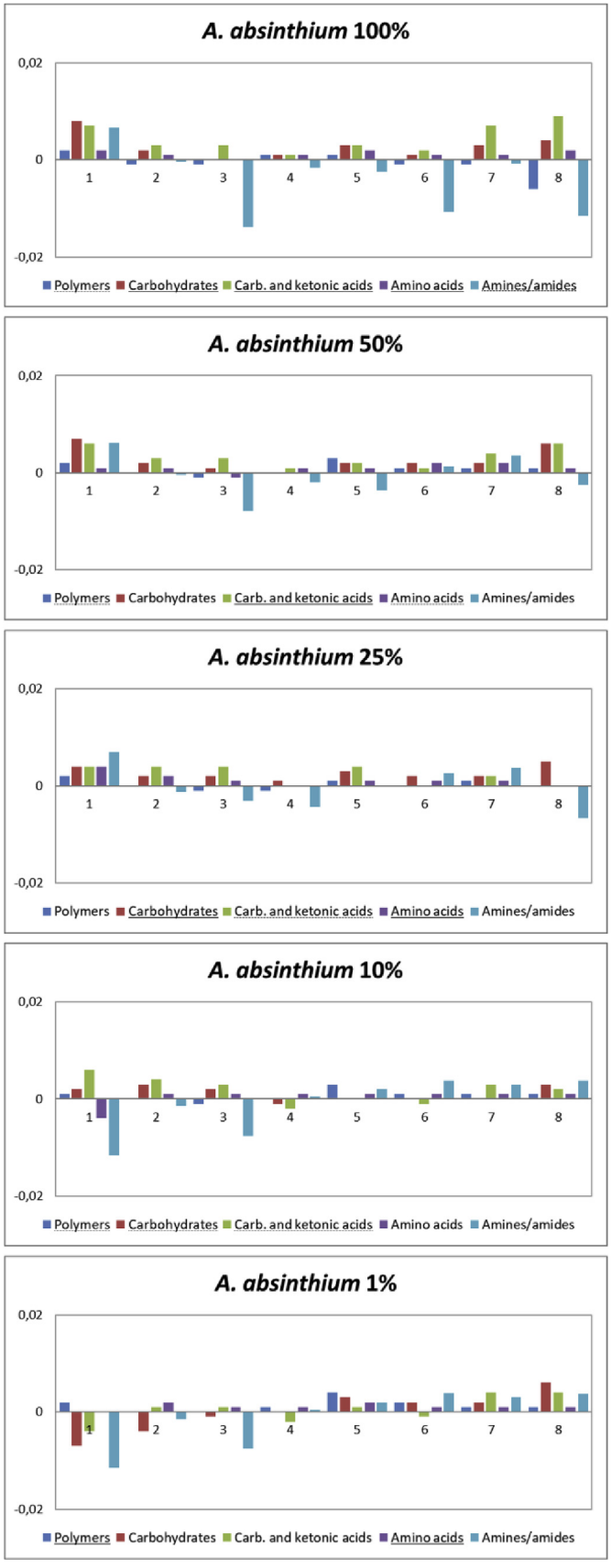

Fig. 7. Bars represent the variation of the Shannon–Weaver index for each group of metabolites, of soil bacteria communities treated with *A. absinthium* L hydrolate during 168 h. Concentrations are in % v/v. Data was measured at intervals of 23 h, 8 times, and values were obtained by subtracting those from the negative control (x-axis values). A continuous line under metabolic groups indicated that differences regarding the control are statistically significant ($p < 0.05$). Lower significance ($p < 0.5$) was indicate by a dashed lines.

apical meristem of *Brassica campestris,* indicating a potential impact on processes involved in cell proliferation (Koitabashi et al., 1997; Nishida et al., 2005). In addition, 1,8-Cineole was found to inhibit both proliferation and elongation of BY-2 Cultured Tobacco Cells (Yoshimura et al., 2011). An explanation for the impacts of this compounds on cell proliferation may rely on the microtubules, a key cell organelle in cell division, that has been reported to be a target for monoterpenes (Chaimovitsh et al., 2017).

However, the mechanism of action of (Z)-2,6-dimethyl-5,7-octadiene-2,3-diol is still unknown.

Terpenoids can cause damage to cell membranes and produce cytotoxicity (Bakkali et al., 2008). The lipophilic property of monoterpenes (Weidenhamer et al., 1993)—as well as the lipid oxidation and deterioration of membrane integrity and permeability in plant cells exposed to monoterpenes (Kaur et al., 2011; Maffei et al., 2001; Zunino and Zygadlo 2004)—suggests that biological membranes, including mitochondrial membranes, may be the primary target of monoterpenes. It is likely the effects on biological membranes when added to the deleterious effects on mitochondrial membranes—which affects energy metabolism—disturbs a wide range of physiological and biochemical processes within the cell (Yoshimura et al., 2011). It is clear that the wall of plant cells in the root do not prevent the action of (Z)-2,6-dimethyl-5,7-octadiene-2,3-diol on cell membranes of the roots of *A. cepa* (Yoshimura et al., 2011).

The acute toxicity of *A. absinthium* hydrolate to *E. fetida* indicates that the product has plenty of bioavailability in the earthworm. The application of the monoterpene as a hydrolate in the soil allows an effective amount of contact with the body of the earthworm. The contaminants present in pore water are available to earthworms via dermal uptake (Vijver et al., 2003). The biochemical composition of the earthworm's cuticle has been well investigated and is extremely tolerant to water uptake and loss (Wallwork, 1983); this potentiates an intense exchange of water across the body wall (Laverack (1963) (Saxena et al., 2014); suggest a mechanism of action dependent upon several insecticides on *E. fetida*. One mechanism involves the binding of biocides with the outer body wall protein/lipid molecules of the earthworm, which can cause damage to the mucopolysaccharide outer layer and cause paralysis of the earthworm. Ingestion of soil particles containing the active products (Suthar et al., 2008) or the direct uptake of active products from solid soil particles could also be another avenue of exposure to the products of the hydrolate (Vijver et al., 2003).

4.2. Effect on microbial soil communities

Although the effects of traditional pesticides on soil microorganisms have been widely studied (Karas et al., 2018; Oleszczuk et al., 2014; Wang et al., 2010), few studies can be found in the literature that analyse the effects of products derived from plants with a pesticidal action on the edaphic microbiota (Gopal et al., 2007; Gupta et al., 2013; Sarawaneeyaruk et al., 2015; Singh et al., 2015; Walvekar et al., 2017). Usually, these studies are focused only on the culturable fraction of the bacterial population, which is only a small fraction of the total bacterial population of the edaphic ecosystem. In this study, the qualitative analysis of bacterial community structures using 16S rRNA led to the elucidation of the impact of the hydrolate on the total soil bacterial population.

Our results showed that dilutions above 25% v/v of the hydrolate

decrease the metabolism of soil bacteria significantly. This is compatible with the effects of other plant-based biopesticides, such as the Azadirachtin (Gopal et al., 2007; Singh et al., 2015; Walvekar et al., 2017) and Neem extract with Azadirachtin (Sarawaneeyaruk et al., 2015). These biopesticides significantly reduced the abundance of the active rhizospheric bacterial population, presenting similar effects to chemical pesticides but at higher doses and less durable effects.

However, at lower concentrations (1% v/v) the hydrolate did not cause inhibition but enhanced metabolism. These stimulatory effects may be explained both by the use of this product as a source of nutrients and by the elimination of bacterial competitors (e.g. fungal populations), as well as the concomitant increase in heterotrophic soil bacteria (Bending et al., 2007; Munoz-Leoz et al., 2011). This effect has also been reported for soil pesticides, such as Glyphosate (Ratcliff et al., 2006) and carbendazim (Tortella et al., 2013).

On the other hand, our results showed that hydrolate caused minor changes on the global metabolic diversity of the soil microorganisms (see Fig. 6). High doses of hydrolate increased the microbial community capacity to metabolize some specific substrates —such as carbohydrate, carboxylic and ketonic acids and amino acids (see Fig. 7) —. The small increases and decreases on the metabolism of the different substrates, compensated the AWCD values, resulting in no differences in metabolic diversity. This “smoothing effect” has been reported for other biopesticides (Walvekar et al., 2017), and also in freshwater bacteria exposed to the same hydrolate (Pino-Otin et al., 2019).

There is an insufficient number of studies about the toxicity mechanisms of the monoterpene (-)-(Z)-2,6-dimethylocta-5,7-diene-2,3-diol for bacteria. However, the deleterious effects of terpenoids have been related to altered cell membrane integrity and sodium channel activity that disturbs the permeability of the biological membranes of the microorganism (Bakkali et al., 2008; Spicakova et al., 2017). Accordingly, the prevalence of Pseudomonas in our soil samples and its resistance to chemicals based on membrane mechanisms (Tórtora et al.) may be the reason behind the small impacts detected in microbial metabolism. Pseudomonas are considered one of the more abundant taxa in soil bacterial communities (Janssen, 2006). In addition, Pseudomonas are characterized because they can use a great diversity of organic compounds as a source of carbon and energy for growth.

In the previous study by Pino-Otin et al. (2019), *Vibrio fischeri*—-which are gram-negative bacteria with high sensitivity to the hydrolate—it was suggested that active compounds may cross the complex cell wall of Proteobacterias, including the outer membrane.

Probably, many of these doubts can be clarified when the mode of action of the biocidal component, 2,6-dimethylocta-5,7-diene-2,3-diol from the *A. absinthium* hydrolate be better understand.

4.3. Environmental relevance

This hydrolate presented nematicidal activity against a target organism *Meloidogyne javanica*, a pathogen nematode for plants, at 33% concentration (Julio et al., 2017b). Our results showed acute toxicity at lower concentrations than that needed to act as pesticide for the three non-target organisms. From lowest concentration to higher: *A cepa* (LC_{50} = 3,87% v/v), *E. fetida* (values of LC_{50} are equivalent to a hydrolate dilution of 20% v/v in water applied to the 600 gr of soil) and soil bacteria in which, at 25% v/v hydrolato concentrations, a significant inhibition of growth occurs.

On the other hand, the nematicidal activity of the hydrolate decreases somewhat over time; after 28 days the hydrolate inhibits still more than 75% of Meloidogyne javanica hatching (Julio et al., 2017b). This agrees with the fact the AWCD values in bacterial test suggest a recover of the system at 7 days (see Fig. 5). This results highlights the need for more detailed studies on the persistence of activity of this extract and its components to confirm that these effects may be less permanent than in the case of traditional pesticides.

5. Conclusion

A. absinthium hydrolate caused acute toxicity for non-target organisms belonging to different trophic levels. The toxicity can be detected at low hydrolate concentrations and in a dose-dependent manner. The hydrolate caused phytotoxicity in *Allium cepa,* leading to a strong inhibition in root growth; it also caused high mortality in the earthworm *Eisenia fetida*. The hydrolate also reduced the bacterial metabolism of a natural soil microbial community, although the physiologic diversity the community analysed through 16S rRNA gene sequencing was only slightly modified. These toxicity effects occur in concentrations lower than those required to control the target organisms. The physical and chemical properties of this main component ((-) - (Z) −2,6-dimethylocta-5,7-diene-2,3-diol), probably make it possible for it to cross biological membranes, which would explain such an intense effect on such diverse soil organisms. These results show that plant-based substances with a pesticidal action are not completely safe for the soil, thus highlighting the need for more studies in natural soils over longer times to ensure that these compounds are safer alternatives to synthetic pesticides.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.05.055.

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