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Pesticide contamination in an intensive insect predator of honey bees

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ABSTRACT

Pesticides used for plant protection can indirectly affect target and non-target organisms and are identified as a major cause of insect decline. Depending on species interactions, pesticides can be transferred into the environment from plants to preys and predators. While the transfer of pesticides is often studied through vertebrate and aquatic exposure, arthropod predators of insects may represent valuable bioindicators of environmental exposure to pesticides. A modified QuEChERS extraction coupled with HPLC-MS/MS analysis was used to address the question of the exposure to pesticides of the invasive hornet *Vespa velutina*, a specialist predator of honey bees. This analytical method allows the accurate quantification of nanogram/gram levels of 42 contaminants in a sample weight that can be obtained from single individuals. Pesticide residues were analyzed in female workers from 24 different hornet nests and 13 different pesticides and 1 synergist, piperonyl butoxide, were identified and quantified. In 75 % of the explored nests, we found at least one compound and in 53 % of the positive samples we could quantify residues ranging from 0.5 to 19.5 ng.g^{-1}. In this study, hornets from nests located in sub-urban environments were the most contaminated. Pesticide residue analysis in small and easy to collect predatory insects opens new perspectives for the study of environmental contamination and the transfer of pesticides in terrestrial trophic chains.

1. Introduction

Pesticides are partly responsible of the current arthropod decline worldwide (Ewald et al., 2015; Goulson, 2019; Outhwaite et al., 2020; Schulz et al., 2021) and have impacts on their predators such as other arthropods, birds and bats (Douglas et al., 2015; Frank and Tooker, 2020; Hallmann et al., 2014; Moreau et al., 2022; Tooker and Pearsons, 2021) through the food chain. Despite efforts made on more precise spraying methods and dose reduction, pesticides are still present on non-target plants and organisms within agricultural fields, field margins (Botías et al., 2016; Brühl et al., 2021; Ward et al., 2022) and in urban environments (Botías et al., 2017; Démares et al., 2022; Md Meftaul et al., 2020). Terrestrial insects can be exposed by feeding or drinking on contaminated sources or by contact with the product or with contaminated surfaces (Gibbons et al., 2015). Most biological control methods used in agriculture are based on the consumption of pests by parasitoids or predatory insects, which are themselves consumed by small mammals and birds. Both pests and their higher predators in the trophic chain are thus likely to be contaminated by pesticide residues with concentrations that might result in the lower efficiency of biological control methods. While exposure pathways and contamination are quite well studied in higher vertebrate predators (Fritsch et al., 2022; Moreau et al., 2022), the contamination processes of invertebrate higher predators are poorly investigated. However, through potential bioaccumulation and biomagnification processes, arthropod preys may represent a source of important contamination for their invertebrate predators.

This study investigates the contamination of an intensive predator of honey bees with pesticides in more or less urbanized environments. When foraging on nectar and pollen, bees are exposed to multiple chemical residues known to induce numerous side effects (Cullen et al., 2019; Iwasaki and Hogendoorn, 2021; Tamburini et al., 2021; Tison et al., 2016) and accumulate in their tissues with time (Rondeau et al., 2015; Tison et al., 2016; Weisbrod, 2020). The Asian hornet (*Vespa velutina*) is an invasive species which intensively predates honey bees in

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Europe (Monceau et al., 2014b). Female workers hornet intensively predate honey bees in front of beehives in summer and autumn, these preys representing the large majority of the diet provided to their larvae after mid-July (Monceau et al., 2014b; Rojas-Nossa and Calviño-Cancela, 2020). Pesticide residues gathered through bee predation could thus accumulate in the larvae and inside the nest and be present as residues in female workers collected later in the season. Exposure to pesticide residues is expected to have similar long-term effects on their cognitive capacities, their behavior and fitness as for honey bees or other pollinators (Balbuena et al., 2015; Goulson et al., 2015; Rundlöf et al., 2015; Tison et al., 2019, 2016).

Beside the direct effects of pesticides themselves, co-formulants (CFs), which facilitate the storage, handling, application and action of the active ingredients (Hazen, 2000), may also play a role in the toxicity and bioaccumulation processes. This is the case with the synergist piperonyl butoxide (PBO); a cytochrome P450 inhibitor (Hodgson and Levi, 1999) often combined with insecticides (carbamates, pyrethrins, pyrethroids, and rotenone) to enhance their toxicity and thus reduce the dose of active ingredient in the formulation (David et al., 2015; Gaweł et al., 2019; Kiljanek et al., 2016; Mullin et al., 2010). Most research focused on the effects of active ingredients reflecting that CFs are considered 'inert' ingredients and non-toxic under EC Regulation No 1107/2009 (Straw et al., 2022). However, the exposure of non-target fauna to such chemicals is of concern since their amount in the plant protection product (PPP) can reach 10 times the amount of the active ingredient. Some CFs have already been found in honey bee matrices (Bishop et al., 2018; David et al., 2015; Johnson et al., 2013; Mullin et al., 2010) and proof that these compounds are not as 'inert' as they appear was noticed by Johnson et al. (Johnson et al., 2013) and then confirmed by several studies (Chen et al., 2018; Mullin, 2015; Straw et al., 2022; Straw and Brown, 2021; Tison et al., 2017). Herein, for the first time, residues of multiple pesticides and a synergist (PBO) were quantified in female Asian hornets collected in wild nests using a modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction coupled with HPLC-MS/MS analysis, originally designed for the multi-class analysis of pesticide residues in fruits and vegetables (Anastassiades et al., 2003) and now widely used for liquid or solid biological matrices (Anastassiades et al., 2003; Mullin et al., 2010; Taliansky-Chamudis et al., 2017). Sample preparation entails acetonitrile extraction with salting-out followed by dispersive solid phase extraction (d-SPE) cleanup (Anastassiades et al., 2003). After extraction, high performance liquid chromatography and/or gas chromatography coupled with mass spectrometry (HPLC-MS and/or GC–MS) are usually used for the sensitive detection and quantification of pesticide residues. One limitation of such extraction methods is the need for sample weights between 1 and 15 g (Kasiotis et al., 2018; Wiest et al., 2011). Although these methods can achieve good levels of sensitivity, they cannot provide information on individual contamination. We modified a method developed by David et al. (2015) and used sample weights as low as 250 mg that can later be used to detect multiple pesticide residues in one single hornet.

The analytical method was optimized and validated for trace analysis of 41 pesticides belonging to 4 pesticide types and a synergist in *V. velutina*. This method was applied for prospecting natural contamination of female hornets collected into their nests in different environments from the Bordeaux region in order to evaluate their potential for biomonitoring. The analysis of soil occupation in these areas was used to link pesticide residues to certain types of environments and crops.

2. Material and methods

2.1. Analytes and reagents

Details of molecules used in the study are given in Supporting Information Table S1. Fungicides and other insecticides commonly used in viticulture which is the major crop in our studied environment (Bordeaux region, South-West of France), were selected including previously highly used molecules known for their persistence but now banned (e.g., atrazine, carbendazim) and other common pesticides frequently found in bee-hive matrices (David et al., 2015; Gaweł et al., 2019; Kiljanek et al., 2016; Mullin et al., 2010).

Analytical standards of 42 molecules and triphenylphosphate (TPP), used as generic internal standard, were purchased from Sigma-Aldrich Chimie (St Quentin Fallavier, France). Isotopically labelled internal standards carbendazim-d_4 (99.3 % isotopic purity), fludioxonil-$^{13}C_2$ (99.6 % isotopic purity) tebufenozide-d_9 (98.9 % isotopic purity) and, tebuconazole-d_6 (100 ng/µL in acetone, 97.5 % isotopic purity) were supplied by Cil Cluzeau Info Labo (Sainte-Foy-La-Grande, France). All pesticide standards were in the range 97–99.9 % chemical purity. LC-MS grade acetonitrile (ACN LC-MS, $\geq$ 99.9 % purity) was obtained from VWR International SAS (Rosny-sous-Bois, France) and glacial acetic acid from Sigma Aldrich (same source as above).

Concentrations of the internal standard working mixture were: TPP 500 ng.mL^{-1}, carbendazim-d_4 545 ng.mL^{-1}, fludioxonil-$^{13}C_2$ 1 µg.mL^{-1}, tebufenozide-d_9 10 µg.mL^{-1} and, tebuconazole-d_6 550 ng.mL^{-1} in ACN LC-MS. The stock solutions of the pesticide mix used for calibration and evaluation of recoveries and precisions were prepared in ACN LC-MS at a concentration close to 20 mg.L^{-1}. Further dilutions were applied to obtain solutions in ACN LC-MS and acetic acid mixture (99:1) at 40, 20, 10, 4, 1, and 0.2 µg.L^{-1} equivalent to 200, 100, 50, 20, 5, and 1 ng.g^{-1} concentrations in hornets. All solutions were stored at − 20 °C in the dark. Anhydrous sodium acetate (NaOAc), magnesium sulfate ($MgSO_4$), primary secondary amine (PSA), bonded silica (C18) and zirconium dioxide-based (Z-Sep +) sorbents were supplied by Sigma-Aldrich (Saint-Quentin-Fallavier, France). Water was purified through a GENPURE UV-TOC system by Thermo Fischer Scientific (Illkirch-Graffenstaden, France).

2.2. Sample collection

Twenty-four nests were collected without any use of insecticides in more or less urbanized environments (see Methods, Statistical analysis) in southwestern France (Gironde department: 18 nests, and Landes department: 6 nests) during summer, autumn or winter (from October 2019 to November 2020). The localization of the nests and date of collection are provided in Supplementary Information Table S2. Once collected, hornets inside the nests were killed by putting the nest in the deep-freezer (-20 °C) and samples were collected and stored at −20 °C before analyses. All *V. velutina* female workers used for the development of the method, calibration curves, and matrix effects originated from the same single nest (nest #4, Supporting Information text and Table S2). Three other nests were used for the evaluation of recovery rates: #15, #16 and #18 (Supporting Information text and Table S2).

2.3. Extraction procedure

For the validation of the method and to limit variations among individuals, five *V. velutina* female workers of similar size from the same nest were put in liquid nitrogen before grinding in stainless steel 10 mL grinding jars (cat. no. 69,985 from Qiagen, Hilden, Germany) for 6 × 30 s at a frequency of 30 Hz (1800 oscillations per minute) using the crusher TissueLyser II (Qiagen, Hilden, Germany). The mash was homogenized, and 250 ± 1 mg of sample was transferred into a 15 mL Falcon tube. For each nest, this was replicated 3 times (triplicate). The same protocol can be applied with single individuals of a minimum dry weight of 300 mg in order to ensure being above 250 mg of sample for the extraction.

First, 1 mL of water and 20 µL of internal standard working mixture (carbendazim-d_4, fludioxonil-$^{13}C_2$, tebuconazole-d_6, tebufenozide-d_9, TPP) were added to the sample to form a suspension. Samples were then extracted by adding 1.25 mL of a solution of acetonitrile (ACN) containing 1 % (v/v) of acetic acid (AcOH) and vortexed for 30 s and

followed by 10 min on a horizontal shaking table (440 rpm). Salting-out was then performed with a 625 mg mixture (4:1) of $MgSO_4$ and NaOAc in turn with immediate hand shaking (10 s) and vortex stirring (15 s) to disperse the salts and prevent magnesium sulfate caking. After 3 min on the shaking table (440 rpm) and centrifugation (4500 rcf for 5 min at 4 °C), 1 mL of supernatant was withdrawn to a 2 mL Supel$^{(TM)}$ QuE Z-Sep + with $MgSO_4$ Tube (Sigma Aldrich, St Quentin Fallavier, France) and immediately hand-shaked for 10 s. The suspension was then vortexed (15 s) and then shaken 10 min on the shaking table and then centrifuged (10 min, 13 000 rcf at 4° C). The supernatant was transferred to a nylon spin filter (0.22 µm pore size, VWR, Rosny-sous-Bois, France) and centrifuged (5 min, 13 000 rcf at 4 °C). Centrifugal filtration in 100 % organic solvent is recommended (David et al., 2015) to avoid analyte losses that can occur when filtering aqueous extracts through the nylon membrane of the filter. The extract was evaporated to dryness in glass tubes using a Speed Vac (Jouan RC 1010) coupled with a Cooling Trap (Jouan RCT90) during 50 min and reconstituted with 150 µL of ACN/H_2O (30:70). The extract was centrifuged for 20 min at 4 °C and 13 000 rcf. In some extracts, a white lipidic layer was observed above the supernatant, and was discarded by collecting the extract from the bottom of the glass tube. Acetonitrile ACN/H2O (30:70) was chosen as reconstitution solvent over more aqueous solvent mixtures as it allows for better solubilization of the more hydrophobic analytes prior to HPLC-MS/MS analyses. Finally, each sample was filtered through a PTFE filter (0.2 µm pore size, 13 mm ø, VWR, Rosny-sous-Bois, France) in an HPLC vial equipped with a 300 µL-insert (Agilent Technologies, Massy, France) using a 1 mL syringe and 0.40 × 12 mm stainless steel needle (Fischer Scientific, Illkirch Graffenstaden, France).

2.4. HPLC-MS/MS analysis

Analyses were performed with a liquid chromatograph (LC) from the 1200 series coupled to a 6430 triple quadrupole mass spectrometer (MS) from Agilent Technologies (Massy, France). The LC consists of a 1260 binary pump, 1260 high performance degasser, 1290 thermostat for the 1260 autosampler, and 1290 thermostated column compartment. The mass spectrometer was equipped with an electrospray ionisation (ESI) source. The system operates with the MassHunter Workstation software version B.05.00.

The column compartment was thermostated at 40 °C. The injection volume was 10 µL and the mobile phase flow was 0.3 mL.min^{-1}. Compounds were separated on a Poroshell 120 EC C18 column (2.1 × 150 mm, 2.7 µm) from Agilent Technologies (Massy, France). Mobile phase A consisted of 0.05 % (w/v) of ammonium formate and 0.01% (v/v) of formic acid in ultra-pure water. Mobile phase B consisted of 0.05 % (w/v) of ammonium formate, 5% (v/v) of ultra-pure water, and 0.01% (v/v) of formic acid in acetonitrile (HPLC-MS quality grade). A solvent gradient was applied for the separation starting with A/B: 95/5, ramped to 100% B linearly over 15 min, and held for an additional 10 min at 100% B. The solvent ratio was then returned to 95/5 (A/B) in 3 min and held for 6 min for column equilibration before the next injection. The total time between two samples was 34 min. The column effluent was diverted to the waste after 25 min run. The retention times of each of the 42 analytes are listed in Supporting Information Table S1.

Nitrogen was produced by a generator NiGen LCMS 40–1 from Gengaz (Wasquehal, France) and used as drying, nebulizing and collision gas. The ESI interface operated simultaneously in positive and negative mode with the capillary voltage set at 3000 V, the nebulizer pressure at 40 psi, the drying gas flow at 11 L.min^{-1} and the gas temperature at 350 °C. The fragmentor voltage had been optimized for each parent ion and the collision energy and cell accelerator voltage had been optimized for each fragmentation. The detection was performed in dynamic multiple reaction monitoring (dMRM) mode and the transitions were sought for 2 min around their retention time.

2.5. Statistical analysis

QGIS Geographic Information System together with Google satellite images and OSO Land Cover Map 2020 (Thierion et al., 2022) were used to categorize soil occupation in a 1 km range around each of the collected nest. The following categories were used: urban (urban areas + roads + industrial zones together), cereals, corn, grassland, orchard, vineyard, forest, water, oilseed). Urban, rural and sub-urban environments were determined by the percentage of urbanization (soil occupation belonging to urban areas, roads, industrial zones) in a 1 km radius around the collected hornet nest (rural: < 20 % urbanization; sub-urban: 20 % < urbanization < 50 % and urban: > 50% urbanization).

A Redundancy Analysis (RDA) was used to test the distribution of pesticides (presence/absence of the compounds) according to the soil occupation (vineyards, urbanization, forests and grassland) in a 1 km radius around the collected nests. Links between the proportion of vineyards and the number of pesticides per type were tested with a Linear Model (LM). All statistics were made using R (version 4.0.1, R Core Team 2020) implemented with *lme4*, and *vegan* package for rda (Oksanen et al., 2022).

3. Results

The analyses revealed 14 different substances out of 42 searched in the collected hornets, including 8 fungicides, 3 insecticides, 2 miticides, and 1 synergist, piperonyl butoxide (PBO) (Supporting Information Table S3, Fig. 1). We did not find any of the 5 targeted herbicides in the collected samples.

The most frequently detected compound was the synergist PBO, which was found in 37.5 % of the analyzed colonies (n = 24), followed by the fungicides hexaconazole (16.6 %) and pyrimethanil (12.5 %). We also detected DMPF (*N*-(2,4-dimethylphenyl)-formamide, CAS [60397–77-5]), the degradation product of amitraz, in 12.5 % of the samples. Amitraz and cymiazole (detected in 4.2 % of the samples) are both miticides used to treat honey bee colonies against the parasitic mite *Varroa destructor*.

From 34 positive detections to at least one pesticide (>LOD), 53 % of detections were above the LOQ and thus could be quantified. Pesticide concentrations above LOQ ranged between 0.5 and 19.5 ng.g^{-1}. Several detected compounds have much higher LOQs than others (Supporting Information Table S1: hexaconazole 12.7 ng.g^{-1}, spiroxamine 20.2 ng.g^{-1} or tebufenozide 48.0 ng.g^{-1}) making them appear in Fig. 1 at concentrations below their LOQ even though they were present at important levels (LODs: 3.8 ng.g^{-1}, 6.1 ng.g^{-1} and 14.4 ng.g^{-1} respectively).

From each collected hornet colony, 15 female hornets were analyzed in triplicate of 250 mg samples. Within the 24 hornet colonies collected, 18 (75%) were positive to at least 1 of the targeted pesticides (Fig. 2). We found an average of 2 pesticide residues per positive sample, with a maximum of 6 different pesticides in a single sample. Six nests contained a mixture of different type of pesticides or synergist, in majority fungicides and PBO.

The highest total pesticide concentration was found in nest #9 collected in a sub-urban environment composed of 23 % urban setting, 39 % grassland, 33 % forest and 5 % water (Fig. 3, Supporting Information Table S2). A mixture of tebufenozide (insecticide, LOD 14.4 ppb), hexaconazole (fungicide, max 17.9 ppb) and PBO (synergist, max 19.5 ppb) was found in this nest, making a total pesticide load three times higher than in the other nests.

We found PBO in 9 of the 24 collected nests. It is present in almost half of the nests collected in sub-urban and urban environments whereas it is absent from the 5 nests collected in rural environments.

Soil occupation in the 1 km surrounding the collected nest was characterized and represented in Fig. 3.

The first model revealed a significant effect of the proportion of vineyards ($F_{1,22} = 3.1515$, $P = 0.0179$) on the probability of finding the compounds presented in Fig. 1 in a 1 km range around the collected

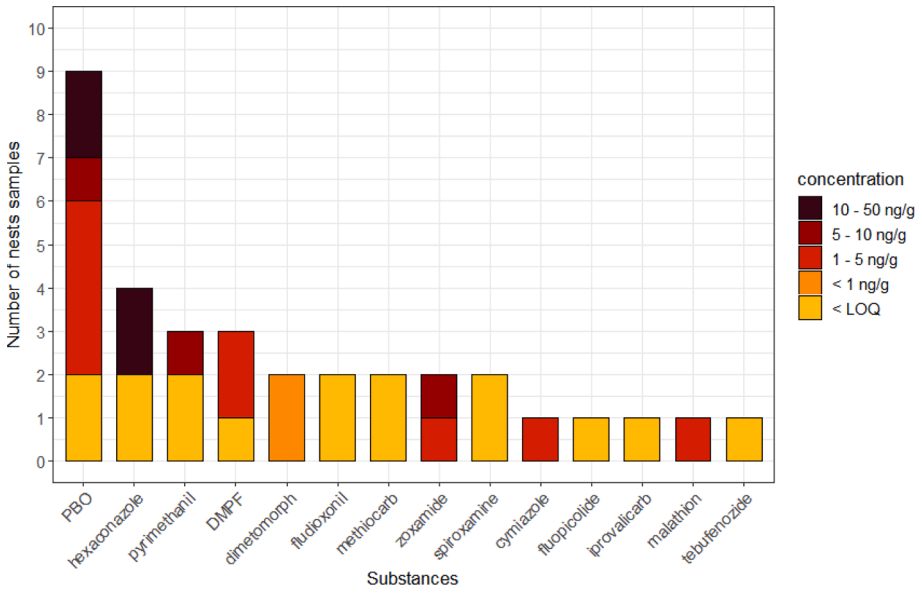

Fig. 1. Number of detections and concentration range (in ng.g^{-1}) of 13 pesticides and PBO in hornet workers (whole bodies) collected in different nests. Detection counts are relative to the individual LODs and LOQs mentioned in Supporting Information Table S1. Values used are the maximum detected concentration in one sample (Supporting Information, Table S3).

nests (Table 1).

When separated by type of pesticides, a significant effect was noticed between the proportion of vineyards and the number of fungicides ($F_{1,22}$ = 5.2009, P = 0.0358) found in the surroundings (1 km) of the nest. There is also a tendency ($F_{1,22}$ = 3.6479, P = 0.0732) of finding more insecticides when the proportion of vineyards in the environment increases.

4. Discussion

Vespa velutina hornets are, so far, the only known intensive insect predators of honey bees. Most of the annual life cycle, the predation is devoted to workers (females) and the preys (quality and quantity) varies along the life cycle of the colony (Monceau et al., 2014b). *V. velutina* female workers start to attack hives in July until autumn and hundreds of hornets can be observed daily on hives (Monceau et al., 2014a). None of the nests analyzed in this study were collected in spring for the reason that hornets born before mid-July could have been fed on more various sources. The majority of the nests were collected in summer and autumn and at this period, >95% of the predating trips are short in time suggesting that female hornets follow an opportunistic pattern by predating on species that can be found in abundance in the vicinity of the nest (Monceau and Thiéry, 2017; Poidatz et al., 2018). During this predation period, hornet larvae are fed by impressive amounts of honey bees. Studying the predation intensity with tagged hunters on a 6 hives apiary revealed that each of the 350 hornets that visited the patch each day were preying up to 4 bees per day during 12 days (Monceau et al., 2014a). Nevertheless, contamination of hornets could additionally occur when hunting other contaminated insects (Rome et al., 2021) or when gathering nectar (Ueno, 2015), water or wood for nest construction.

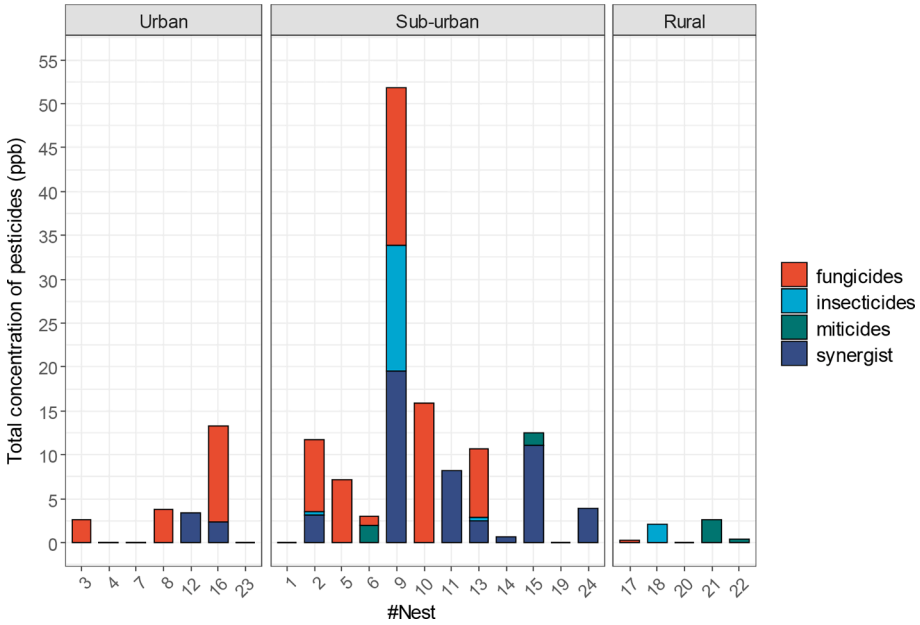

Fig. 2. Pesticide residue distribution and concentration in hornet samples from nests collected in different environments. Values used are the maximum detected concentration in one sample (Supporting Information Table S3). Concentrations over the limits of detection (≥LOD) but below the limits of quantification (<LOQ) were assigned the LOD value. ppb = ng.g^{-1} ^{ng}/gram wet weight of sample. Soil occupation: rural < 20 % urbanization; sub-urban 20 % < urbanization < 50 % and urban > 50% urbanization.

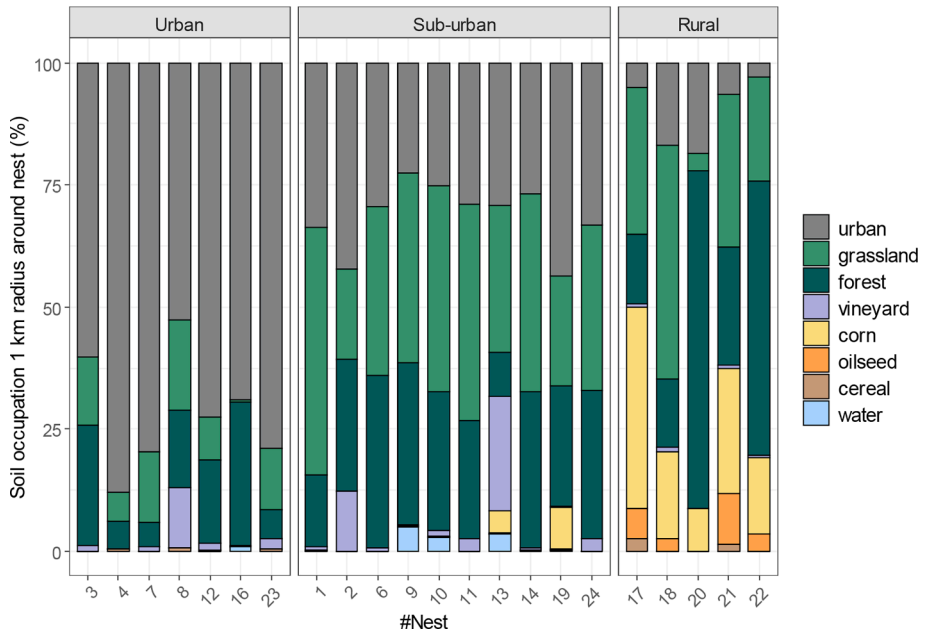

Fig. 3. Soil occupation in a 1 km range around each collected nest. Land use of the different environments was obtained from OSO Land Cover Map 2020 (Thierion et al., 2022). Soil occupation: > 50 % urbanization = Urban, > 20 % and < 50 % urbanization = Sub-urban, < 20 % urbanization = Rural.

Table 1
Influence of soil occupation on pesticide residues found in hornets.

RDA compounds ~ soil occupation			*LM* vineyards ~ types of pesticide		
	F	P-value		F	P-value
forests	1.2494	0.2682	**fungicides**	**5.2009**	**0.0358**
vineyards	**3.1515**	**0.0164**	insecticides	3.6479	0.0732
urban	1.4596	0.1853	PBO	0.1136	0.7402
grassland	1.0780	0.3598	miticides	0.0263	0.8730

However, nectar and fruit consumption only occurs at the adult stage, and should be viewed as anecdotic as compared to being fed as larvae several times a day during ca. 1.5 month with contaminated insects, which are in large majority bees in summer and autumn. This was unfortunately impossible to quantify in this study but future analyses of larvae and nesting material should bring further information on this matter. Pesticide residues found in urban and sub-urban areas indicate that contaminated nectar and honey bees are present in such areas (Botías et al., 2016; Démares et al., 2022; Mahé et al., 2021), demonstrating the importance of studying urban environments in ecotoxicology because of garden and domestic pesticides use (Md Meftaul et al., 2020). Contaminated honey bees brought back to the nest in order to feed larvae may induce bioaccumulation of pesticide residues in the next generation of hornets and inside the nest. An increase in concentration of pesticides from preys to predators was previously described (Deribe et al., 2013; Rudd et al., 1981). However, the presence of the same compounds in sympatric honey bee and Asian hornet populations needs to be investigated in order to get deeper into the understanding of bioaccumulation and biomagnification in such a predatory chain. If biomagnification should occur in this trophic-chain example, one might expect higher concentrations of certain types of pesticides in other predators of honey bees and hornets such as birds (i.e., *Parus major, Pernis apivorus, Lanius collurio*) but this was not investigated in the present study.

In order to guarantee yields and quality of grapes, viticulture uses large amounts of phyto-pharmaceutical products. The Bordeaux region is known for its wine production consequently leading to important pesticide contamination in the environment. We found that the proportion of vineyards in a 1 km range around the collected nests increased significantly the chance of finding pesticides in the hornets. Fungicides are the most frequent type of pesticides used in viticulture and they are found predominantly in the analyzed nests followed by insecticides. The presence of multiple pesticide residues in hornets at the time of analysis (Fig. 1, Fig. 2) is concerning since synergistic effects of certain pesticides in honey bees were already reported (Schmuck, 2004; Siviter et al., 2021; Tosi and Nieh, 2019; Zhu et al., 2017).

Furthermore, we found the CF piperonyl butoxide in more than half of the nests collected in sub-urban environments, showing the widespread use of pesticides containing PBO in these areas.

PBO is a synergist used in a wide range of formulations including dusts, emulsifiable concentrates, foggers, sprays, etc. (Lewis et al., 2016) by farmers but also by citizens in gardens or houses. PBO increases the effect of several insecticides, e.g., pyrethrum and pyrethroids (Tozzi, 1999) by inhibiting the activity of P450 enzymes (Hodgson and Levi, 1999). Environmental contamination with these substances is thus probable in locations where PBO was found but their quantification is lacking in this study because these insecticides are better analyzed with GC–MS. The mode of action of PBO on detoxifying enzymes might likely penalize hornet and honey bee immune defense system in the same way as it does for pests. Miticides used in beehives against *Varroa destructor* are rapidly metabolized by honey bee P450s contrary to the mite (Johnson et al., 2013). However, recent studies show that PBO might inhibit enzymes that participate in the detoxification process of amitraz (Dadé et al., 2020). The simultaneous presence of PBO and amitraz metabolite (e.g. nest #15) underlies the possibility of such mixture of products in hornet preys. Amitraz metabolites were found in honey samples (Pohorecka et al., 2012) and PBO was recently found with 100 % occurrence in Swiss beeswax samples (Marti et al., 2022) and Italian beeswax (Perugini et al., 2018, p. 201). Interestingly, safety data sheet of amitraz veterinary product advices to avoid the simultaneous use of PBO because of a supposed reduction of the therapeutic activity of the miticide.

The bioconcentration factor (BCF) of PBO (260) is exceeding the threshold for concern in *Lepomis macrochinis* (Lewis et al., 2016) and could be problematic for honey bees or other insects as well. Indeed, biomagnification potential is high with lipophilic substances such as PBO, likely to concentrate in tissues with high lipid content present in most insects including our study model, hornets.

If regulatory bodies were to mandate residue analysis for all agrochemicals, including so-called 'inert' ingredients, one would have a better understanding of the complex exposure terrestrial insects are facing. It is fundamental in risk assessment to consider active substance and co-formulant bioaccumulation and biomagnification potential before considering using them for agricultural purposes (Maurya, 2016).

The method we have developed allows the extraction and quantification of 41 pesticides and a synergist (PBO) in 250 mg samples of Asian hornets *V. velutina* with good recoveries and sufficient level of sensitivity ($ng.g^{-1}$) to quantify low environmental contaminations. The main difficulty was due to the matrix involved. The use of zirconium dioxide-based sorbents (Z-Sep +) allowed additional cleanup of lipids, simplified the methodology and led to better recoveries (Gaweł et al., 2019; Kiljanek et al., 2016). It is a common ground that HPLC–MS/MS is prone to strong matrix effect which may result in signal suppression or signal enhancement. The diversity of chemical and physical properties of the compounds analyzed in multi-class and multi-residue extraction procedures often leads to heterogeneous matrix effects (Roca et al., 2014). In order to have reliable and accurate quantification of pesticide residues, matrix-matched calibration standards or procedural calibration standards (Roca et al., 2014) should be used and the analytes quantified by comparing peak areas in samples and in procedural calibration lines (samples spiked at 1, 5, 20, 50, 100, 200 $ng.g^{-1}$). Matrix effect can also be compensated using isotopically labeled internal standards (IS), such as in this study. Pooled samples were used for the development of this method in order to limit variations and allow replicates. However, a sample weight of 250 mg after grinding can be obtained from a single hornet. In future studies, this method can be used for the extraction of pesticide residues from single individuals, which will provide useful information on the individual contamination of a predator.

The quantification of pesticide residues in a predatory chain such as honey bees and the Asian hornet *V. velutina* is building the foundation for pesticide residue analysis in other terrestrial arthropod trophic chains. The trophic transfer and bioaccumulation of pesticides in terrestrial ecosystems and especially in insects has been neglected in ecotoxicology studies but could provide valuable information on environmental contamination.

In this context, the existence of these pesticides in the environment could pose threat to other organisms than bees or hornets through direct exposure or the consumption of contaminated preys. A fair amount of biological control agents used in agriculture are based on the consumption of pests by natural enemies. Pests contaminated with sublethal doses of pesticides often become resistant to treatments and more sensitive to predation (Tan et al., 2014), thus increasing the risk of pesticide transfer into the food chain. Understanding the transfer and bioaccumulation potential in a predator will help to evaluate the risk of pesticide transfer between biotic compartments and the unexpected possible reduction in the efficiency of biological control methods.

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CRediT authorship contribution statement

Léa Tison: Conceptualization, Investigation, Methodology, Formal analysis, Validation, Visualization, Data curation, Supervision, Funding acquisition, Writing – original draft. **Céline Franc:** Conceptualization, Investigation, Methodology, Formal analysis, Validation, Resources, Writing – review & editing. **Louisiane Burkart:** Methodology, Investigation, Writing – review & editing. **Hervé Jactel:** Conceptualization, Writing – review & editing. **Karine Monceau:** Conceptualization, Writing – review & editing. **Gilles de Revel:** Conceptualization, Resources, Writing – review & editing. **Denis Thiéry:** Conceptualization, Investigation, Project administration, Supervision, Funding acquisition, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2023.107975.

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