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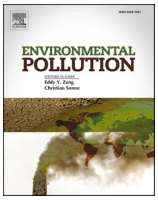

Environmental monitoring study of pesticide contamination in Denmark through honey bee colonies using APIStrip-based sampling☆

María Murcia-Morales [a], Francisco José Díaz-Galiano [a], Flemming Vejsnæs [b], Ole Kilpinen [b], Jozef J.M. Van der Steen [c], Amadeo R. Fernández-Alba [a,*]

[a] Chemistry and Physics Department, University of Almeria, Agrifood Campus of International Excellence (ceiA3), 04120, Almería, Spain
[b] Danish Beekeepers Association, Fulbyvej 15, 4180, Sorø, Denmark
[c] Alveus AB Consultancy, Kerkstraat 96, 5061 EL Oisterwijk, The Netherlands

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ABSTRACT

Due to their extensive use in both agricultural and non-agricultural applications, pesticides are a major source of environmental contamination. Honey bee colonies are proven sentinels of these and other contaminants, as they come into contact with them during their foraging activities. However, active sampling strategies involve a negative impact on these organisms and, in most cases, the need of analyzing multiple heterogeneous matrices. Conversely, the APIStrip-based passive sampling is innocuous for the bees and allows for long-term monitorings using the same colony. The versatility of the sorbent Tenax, included in the APIStrip composition, ensures that comprehensive information regarding the contaminants inside the beehive will be obtained in one single matrix. In the present study, 180 APIStrips were placed in nine apiaries distributed in Denmark throughout a six-month sampling period (10 subsequent samplings, April to September 2020). Seventy-five pesticide residues were detected (out of a 428-pesticide scope), boscalid and azoxystrobin being the most frequently detected compounds. There were significant variations in the findings of the sampling sites in terms of number of detections, pesticide diversity and average concentration. A relative indicator of the potential risk of pesticide exposure for the honey bees was calculated for each sampling site. The evolution of pesticide detections over the sampling periods, as well as the individual tendencies of selected pesticides, is herein described. The findings of this large-scale monitoring were compared to the ones obtained in a previous Danish, APIStrip-based pilot monitoring program in 2019. Samples of honey and wax were also analyzed and compared to the APIStrip findings.

1. Introduction

The use of pesticides has acknowledged benefits in conventional agricultural production and plays an important role in the improvement of the quality and productivity, as well as in the reduction of the crop losses (Aktar et al., 2009). However, the presence of pesticides in the environment may lead to major hazards to human and animal health and impact the ecosystems and, thus, strict controls must be imposed by governments and international authorities to ensure a sustainable, safe use. The Danish Government takes an active part in the control of the manufacture, commercialization and use of pesticides, which is reflected in the substantial decrease in the sales and application of plant protection products in the last years. Between 2011 and 2019, the sales of pesticide products decreased a 52% in Denmark, whereas their application decreased a 22% in the same period (Miljøstyrelsen, 2019). Moreover, in 2017, the Ministry of Environment and Food of Denmark published a detailed four-year action plan aimed at reducing the pesticides load and ensuring a sustainable use of these substances (Danish National Action Pl, 2017). However, the use of pesticides is still a reality and a monitoring of water samples performed in 2019 revealed the presence of more than 30 different pesticides and veterinary products in various locations in Denmark (Casado et al., 2019). Previous studies had already reported the presence of pesticides and contaminants in environmental samples from the country (Asman et al., 2005).

An alternative approach to the assessment of the environmental pollution involves the indirect sampling through honey bee colonies.

☆ This paper has been recommended for acceptance by Da Chen.

* Corresponding author. University of Almeria, Agrifood Campus of International Excellence (ceiA3) Department of Hydrogeology and Analytical Chemistry, Ctra. Sacramento S/Nº, La Cañada de San Urbano, 04120, Almería, Spain.
E-mail address: amadeo@ual.es (A.R. Fernández-Alba).

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These pollinators come into close contact with a wide range of contaminants during their foraging activities and, consequently, these compounds enter the beehives and can circulate and accumulate in apicultural matrices (Rortais et al., 2005; Lozano et al., 2019). This approach has been successfully applied to monitor the presence in the environment of pesticides (Lozano et al., 2019; Kammoun et al., 2019; Murcia Morales et al., 2020a,b), PAHs (Cochard et al., 2021), lead (Lambert et al., 2012) and other metals (van der Steen et al., 2012) or microplastics (Edo et al., 2021), among others. Despite the usefulness of honey bee colonies as sentinels for the presence of contaminants in the environment, this approach typically involves the active sampling of apicultural matrices such as wax, honey, beebread or even honey bees. This results in a) the existence of multiple matrices with different physicochemical properties and, therefore, an irregular distribution of contaminants accumulated (Lozano et al., 2019); b) a limitation in the number of subsequent sampling that can be performed on a single beehive, determined by their availability; c) ethical concerns related to the use of living organisms or their resources for research. All these issues are avoided in the APIStrip-based sampling, a methodology recently developed by our research group as a part of the INSIGNIA project (SANTE/2018/E4/SI2.788418-SI2.788452) (Murcia Morales et al., 2020a,b). The APIStrips are passive samplers based on the sorbent Tenax TA, whose versatility allows for the detection of a wide range of substances. Tenax has been typically employed for the sorption of contaminants in air or sediment samples (Patil and Lonkar, 1994; Lydy et al., 2015), and its solubility in chlorinated solvents such as dichloromethane increases its possible applications (Alfeeli et al., 2010). In this context, APIStrips (Adsorb-Pesticide-Inhive Strip) consist of a thin polystyrene layer covered with a uniform Tenax film that are easily placed inside beehives and that bind contaminants circulating within the colony (transported by the bees). These samplers have shown to provide comprehensive information of the contaminants inside beehives –i.e. the environmental contamination-in one single, representative matrix (Murcia Morales et al., 2020a,b). They have been successfully applied in a pilot monitoring study of pesticides in Denmark and other European countries (in the INSIGNIA framework) and also to the determination of dissipation and cross-contamination of miticides in apiculture (Murcia-Morales et al., 2021). The sampling methodology is quick and easy, and avoids the need of taking apicultural samples, thus minimizing the human impact on the colonies and making it possible to perform multiple subsequent samplings to a single beehive.

The present work is aimed at applying APIStrip-based sampling in a large-scale monitoring study in Denmark, to assess the use of pesticides and their presence in the environment. Ten-biweekly sampling rounds took place between April and September 2020 in 18 beehives (nine selected sampling sites, with two honey bee colonies per apiary). To the best of our knowledge, this is the first large-scale study aimed at monitoring the presence of pesticides in the environment through honey bees in Denmark.

2. Experimental

2.1. Reagents and materials

All high-purity pesticide standards were obtained from LGC (Teddington, United Kingdom), Sigma-Aldrich (Steinheim, Germany) or Riedel-de-Haën (Seelze, Germany) and were stored at −30 °C. Individual pesticide stock solutions (1000–2000 mg/L) were prepared in acetonitrile and stored in amber screw-capped glass vials in the dark at −20 °C. Individual standard solutions, used for optimization, along with standard-mix solutions, used for calibration, were prepared from the stock standards.

Optima LC-MS grade water was obtained from Fisher Scientific (Fair Lawn, NJ, USA). LC-MS grade methanol and ethyl acetate for pesticide residue analysis were purchased from Fluka Analytical (Steinheim, Germany). Ultra-gradient HPLC-grade acetonitrile was obtained from Merck (Darmstadt, Germany).

2.2. Location and management of the apiaries

Nine test apiaries were located in representative areas of Denmark regarding types of landscape and agricultural use (Fig. 1) (Levin, 2019). Denmark is a farming country with more than 60% of the landscape used for farming: the soil in the east is regarded as very rich for farming and in the western part it is sandier and, therefore, plant production is more frequent in the eastern part whereas cattle and milk production is more common in the western part (Levin, 2019). Pig farming is dominant over the country. In the southern part of the islands seed production, especially white clover, is dominant as well as fruit production.

Styrofoam boxes where employed in the colonies, with wax from either closed wax clubs or green wax pools (where there is general agreement on not using pesticides of any type in the colonies). Only organic apicultural practices approved for organic beekeeping were used: for varroa treatment, oxalic acid and formic acid were the only treatments applied. Additionally, thymol (also allowed in organic apiculture) was employed by one of the apiculturists. All of these substances have been found to be a natural part of honey. All colonies where wintered on one box with ten frames. During the season, the colonies where run as classical beekeeping for honey production and swarm prevention; no swarming was reported. All colonies had hybrid bees of the type Buckfast.

All wax within the colonies brood box is replaced at least every second year. The colonies were fed with 18–22 kg of sugar for wintering, and feed was removed in the spring just before the nectar flow started. The season 2020 ended out as a medium to good beekeeping season.

All the Citizen Scientists (CS) were handpicked and regarded as rigorous and skilled beekeepers (Gratzer and Brodschneider, 2021). All of them had previously taken part in a former pollen and propolis project, so they were well educated in collecting samples. Prior meetings, online meetings and personal visits by a study coordinator ensured the compliance with the sampling protocols.

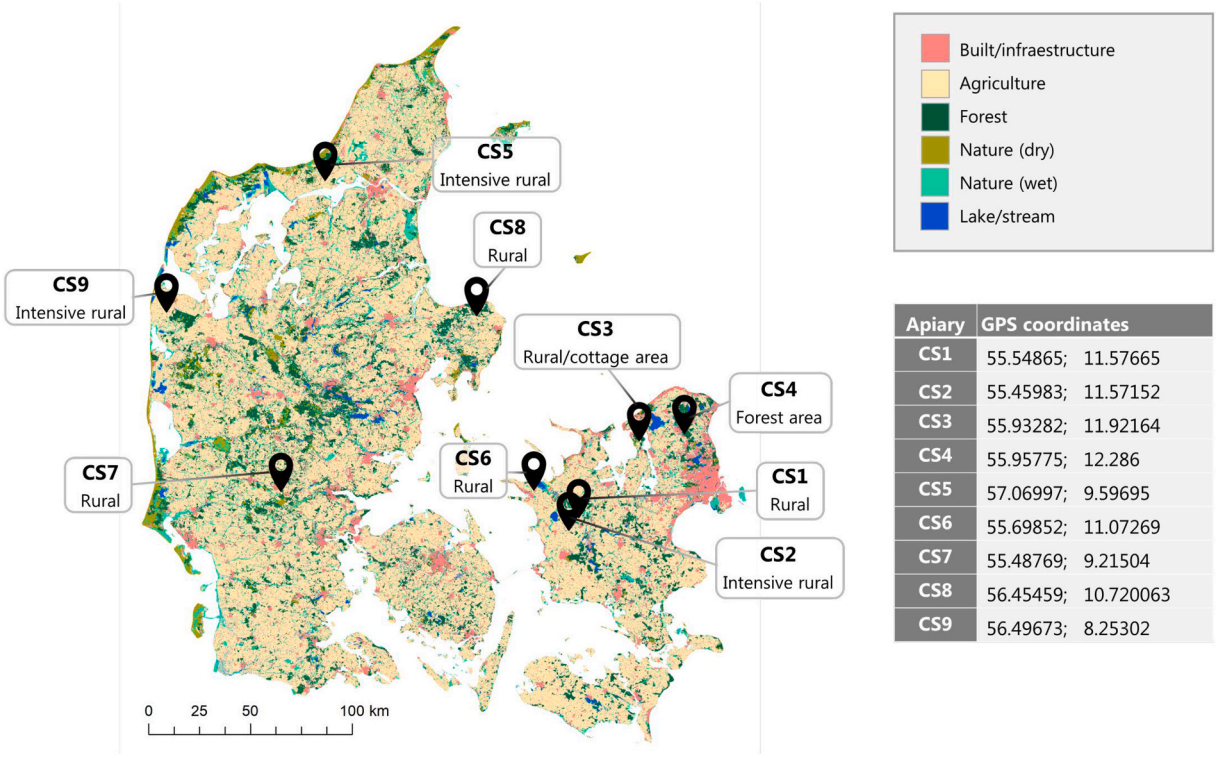
2.3. APIStrip preparation and sampling

The preparation of the APIStrip sampling devices is fully described in a previous study (Murcia Morales et al., 2020a,b). Both sides of a thin polystyrene plastic layer (5 × 10 × 0.2 cm) were covered with 6 mL of a Tenax solution in dichloromethane at high concentration (125 mg/mL). After the evaporation of the solvent under a gentle nitrogen current, the upper section of the strip is drilled to form a small hole. The resulting APIStrip contains 0.75 g of Tenax (0.375 g per side) in a uniform film covering the sampler surface. A thread or wire is then introduced inside the hole to facilitate the introduction of the APIStrips inside the beehive, where they remain for fourteen days (sampling period).

Ten consecutive bi-weekly sampling rounds took place in 2019 and in 2020 in different apiaries in Denmark. The first APIStrips were placed in mid April (April 19th, both in 2019 and 2020) and the first sampling round took place in the beginning of May (May 3rd, 2020 and May 5th, 2019). On each subsequent sampling round, the previous APIStrip was removed from the colony and a new one was inserted for another two weeks. This way, the last sampling round took place in the beginning of September (Sep 6th, 2020 and Sep 8th, 2019).

2.4. Sampling of honey and wax

Two honey samplings took place during the 2020 season: the first one was during APIStrip sampling round 3 (when the rapeseed flow stopped), on May 31st, and the second one, at the end of the sampling season (APIStrip sampling rounds 6 to 9, depending on the availability of honey and before the last honey harvest). Only one of the two colonies from the beekeepers was involved in each honey sampling: colony 1 in the first sampling, colony 2 in the second one. Samples were taken from



Apiary	GPS coordinates
CS1	55.54865; 11.57665
CS2	55.45983; 11.57152
CS3	55.93282; 11.92164
CS4	55.95775; 12.286
CS5	57.06997; 9.59695
CS6	55.69852; 11.07269
CS7	55.48769; 9.21504
CS8	56.45459; 10.720063
CS9	56.49673; 8.25302

Fig. 1. Land use of Denmark and location of the nine sampling sites (CS1 to CS9), with their corresponding coordinates.

fresh honey frames, which the beekeeper could see filled with honey recently. Afterwards, the frames were scraped, so that the honey would flow into the sampling jar.

Additionally, at the end of the sampling season (September 2020), beekeepers cut at a minimum of 10 g of wax out of the frames, which had been in the colony for all the season.

2.5. APIStrip extraction procedure

The desorption procedure of the pesticides from the Tenax film in the APIStrip surface was performed as described in a previous study (Murcia Morales et al., 2020a,b): first, the APIStrips were cut in small pieces and placed inside 50-mL PTFE centrifuge tubes. Then, 10 mL of acetonitrile were added and the samples were automatically shaken at 1250 rpm (Geno/Grinder, 2010; SPEX) for 3.5 min and centrifuged at 4000 rpm (3113 g) for 5 min. This extraction procedure entails a 10-fold dilution (1 APIStrip per 10 mL acetonitrile), which was undone during the preparation of the injection vials.

Procedural internal standards were employed to control the extraction performance: dichlorvos-D6, malathion-D10, carbendazim-D3 and triphenyl phosphate (TPP). The recovery of dichlorvos-D6, malathion-D10 and carbendazim-D3 was checked by LC-MS/MS and the recovery of malathion-D10 and triphenyl phosphate was tested by GC-MS/MS.

For the preparation of the injection vials, 500 μL of the sample extracts were evaporated under a gentle nitrogen stream and reconstituted with 50 μL acetonitrile and 200 μL ultrapure water (LC-MS/MS) or 50 μL ethyl acetate (GC-MS/MS). In both cases, the vial preparation entails a 10-fold concentration (the addition of water to the LC vials does not affect the quantitation). Injection internal standards (dimethoate-D6 for LC, lindane-D6 for GC) were employed to check the variations in the injection volume.

Calibration curves were prepared as follows: a blank APIStrip extract (500 μL) was evaporated and reconstituted with an organic solvent (50 μL ethyl acetate for GC-MS/MS, 50 μL acetonitrile for LC-MS/MS) containing a mixture of pesticides at 0.5, 1, 5, 10, 50, 100 or 200 μg/L. For the LC vials, 200 μL of ultrapure water were added.

2.6. Honey and wax extraction procedure

The extraction of honey and wax samples was performed following the QuEChERS extraction method with a thermostatted automatic shake, as described in previous studies (Lozano et al., 2019). A 10 g portion of homogenized sample was weighed in a 50-mL PTFE centrifuge tube and 10 mL of acetonitrile were added. The samples were shaken in an automatic axial extractor (AGYTAX®, Cirta Lab. S.L., Spain) for 4 min. Then, 4 g MgSO4, 1 g NaCl, 1 g trisodium citrate dihydrate and 0.5 g disodium hydrogencitrate sesquihydrate were added and the samples were shaken in the automatic axial extractor for 5 min using the holder temperature at 40 °C. The extracts were then centrifuged (4500 rpm, 3923 g) for 5 min. A 5 mL volume of the supernatant was transferred to a 15-mL PTFE centrifuge tube containing 750 mg of $MgSO_4$ and 125 mg of PSA. The samples were shaken in a vortex for 30 s and centrifuged again (3500 rpm, 2374 g) for 5 min. Finally, the extracts were transferred to amber vials and they were acidified with 10 μL of formic acid 5% per mL of extract. Procedural internal standards (dichlorvos-D6, malathion-D10, carbendazim-D3 and TPP) were used as surrogate standards to control the extraction performance.

During the preparation of the sample vials, different procedures were followed according to the analytical technique. For GC-MS/MS, 50 μL of the honey or wax extracts were evaporated under a gentle nitrogen stream and reconstituted with 50 μL of ethyl acetate. For the LC-MS/MS injection vials, 100 μL of the extract were diluted with 400 μL of water. Dimethoate-D6 and lindane-D6 were used as injection internal standards in all vials for LC and GC, respectively. Matrix-matched calibration curves were preparing by evaporation of a blank honey or wax extract (50 μL for GC-MS/MS, 100 μL for LC-MS/MS) and reconstitution with the same volume of an organic solvent (ethyl acetate for GC-MS/MS, acetonitrile for LC-MS/MS) containing a mixture of pesticides at 0.5, 1, 5, 10, 50, 100 or 200 μg/L. Finally, 400 μL of ultrapure water were added to the LC-MS/MS injection vials.

2.7. Optimization of compounds

For the optimization of the MS parameters, the 428 pesticide residues included in the study were monitored in full-scan mode in the 50–550 *m/z* range. The first step was the selection of the precursor/s ion/s for each analyte and the retention time, injecting individual solutions for each pesticide at 1 mg/kg in full-scan mode. The ion with the highest intensity and *m/z* relationship was selected as the precursor ion. Precursor ion fragmentation was performed by collision-induced dissociation with nitrogen, from which the best fragment ions were chosen. The most intense transition was selected as the quantifier transition (SRM1), while the second most intense was chosen as the qualifier transition (SRM2). The adequate CE for each transition was assayed in the 3–40 eV range. Retention times, transitions and CEs for each analyzed compound were detailed in a previous study (Ucles et al., 2017).

2.8. GC-QqQ-MS/MS analysis

The analyses by gas chromatography were performed in an Agilent Intuvo 9000 GC system (Agilent Technologies, Palo Alto, CA, USA) equipped with an Agilent 7693 autosampler and an Agilent 7010 B GC-MS/MS triple quadrupole. Data acquisition and processing was developed by Agilent MassHunter QQQ Acquisition and Quantitative Analysis software version 10.0. Samples were injected using a multimode injector inlet in splitless mode, through an Agilent ultra-inert inlet liner with glass wool frit. The injection volume was 1 μL. The injector temperature was kept at 80 °C during the solvent evaporation stage (0.1 min) and then ramped up to 300 °C at 600 °C/min for 5 min and up to 250 °C at 100 °C/min. Two planar columns (Agilent), HP-5MS UI 15 m long × 0.25 mm i.d. × 0.25 μm film thickness were used.

The oven temperature program was as follows: 60 °C for 0.5 min, up to 170 °C at 80 °C/min and finally up to 310 °C at 20 °C/min. The total run time was 12.4 min with 2.1 additional min for backflushing at 310 °C. The instrument worked at a constant flow (1.28 mL/min column 1, 1.48 mL/min column 2). The system worked in dynamic MRM, acquiring the transitions in a ±0.2 min window from the retention time of each analyte. Helium (99.999% purity) was used as the carrier and quenching gas, and nitrogen (99.999% purity) as the collision gas. The collision and quenching gas flows were 1.5 mL/min and 2.25 mL/min, respectively. Both the transfer line and the ion source -operated in electron ionization-were maintained at 280 °C. The quadrupole analyzer temperature was fixed at 150 °C. The solvent delay was 2.6 min.

2.9. LC-QqQ-MS/MS analysis

An Agilent UPLC 1290 Series coupled to an Agilent 6490 Triple Quad LC/MS was used for this study. Data acquisition and processing was developed by Agilent MassHunter QQQ Acquisition and Quantitative Analysis software version 10.0, using dynamic MRM software features with a retention time window of 0.8 min. The injection volume was 5 μL, and the chromatographic separation was carried out with a Zorbax Eclipse Plus C8 column (Agilent), 2.1 mm × 100 mm × 1.8 μm. The system employed 0.1% formic acid in milliQ water (mobile phase A), and 0.1% formic acid and 5% water in acetonitrile (mobile phase B) with the following gradient: 20% of B for 2 min, a linear gradient up to 100% of B in 13 min and finally an isocratic mode at 100% of B for 2 min. Subsequently, an equilibration step coming back to 20% of B (2.5 min) was performed. The system was provided with a JetStream electrospray ion source, employing nitrogen as the nebulizer gas. This ion source was configured as follows: 120 °C for the drying gas temperature, 13 L/min for the drying gas flow rate, 45 psi for the nebulizer pressure, 375 °C for the sheath gas temperature and 10 L/min for the sheath gas flow rate. The MS used nitrogen as the collision gas (99.999% purity), 380 V for the fragmentor and 3000 V for the capillary voltage, both in positive and negative mode.

3. Results and discussion

3.1. Environmental monitoring 2020

3.1.1. General assessment

The monitoring performed in Denmark in 2020 involved the participation of nine apiculturists (Citizen Scientists, CS) distributed throughout the country. Each participant provided two colonies of the same apiary for the passive, APIStrip-based sampling. A total of ten bi-weekly sampling rounds took place (April to September), thus resulting in 180 APIStrips employed and analyzed. Table 1 summarizes the pesticide findings in these samples, sorted by frequency of detection. The average concentration of these pesticides is also shown (in ng/APIStrip), together with their legislative status by the European Commission (EC), in terms of approval for their use in agriculture in the member states. When available, the expiration date of the approval in the non-authorized pesticides was as well included.

Seventy-five different residues were identified in the APIStrips, with 548 total detections (average 3 pesticide residues detected per APIStrip, up to 19 different residues detected in a single APIStrip). Half of these pesticides (50.6%) had only sporadic detections, with just one or two detections throughout the sampling period. Boscalid and azoxystrobin were the most frequently detected compounds, present in 23–28% samples. In the last years, boscalid has been officially reported in a wide variety of crops produced in Denmark, including strawberries, kales, beans, cereal grains, carrots, pears or spinach among many others (O, Boscalid (221) - Eva; Pesticidrester, 2019). This active substance was among the ten best-selling pesticides in Denmark during 2019, with more than 43000 kg sold (Miljøstyrelsen, 2019), which explains the large number of detections in APIStrip samples. Similarly, azoxystrobin was as well detected in a large number of samples from the official monitoring for pesticides in food in 2019, including fruits, vegetables and cereals produced by the Danish agriculture (Pesticidrester, 2019). Also, a previous study reported the presence of azoxystrobin, boscalid, propiconazole, carbendazim, diuron or propyzamide, among others, in water samples from the Danish environment (Casado et al., 2019). For their part, carbendazim, demeton-S-methylsulfoxide, diazinon or omethoate have been considered to be among the 20 pesticides that contribute most to the Hazard Index for the cumulative dietary exposure to Danish adult population (Jensen et al., 2015); these pesticides were detected in up to 18 APIStrips at an average concentration lower than 1.5 ng/APIStrip.

The very high average concentration (more than 1000 ng/APIStrip) of thymol can be explained due to its use as a veterinary treatment for varroa infestations: apiculturist 2 employed thymol strips throughout the sampling period. Something similar happened with two benzalkonium chlorides (BAC8 and BAC10): these compounds, typically employed as preservatives and antimicrobial agents, are included in the formulation of daily products and hydroalcoholic solutions, and they were found at high concentrations in up to 11% of the APIStrips analyzed (*data not shown*). Their presence might be explained on the basis of contaminations of the APIStrip surface with the beekeepers' hands, after the topic application of hydroalcoholic solutions as a prevention for SARS-CoV-2 during the coronavirus pandemic. These findings were not included in the environmental assessment.

As can be observed in Table 1, the majority of pesticides detected in 2020 had a fungicidal or insecticidal activity (32 and 21 residues, respectively). Some of them, such as the frequently detected imidacloprid, demeton-S-methylsulfoxide or chlorpyrifos, possess a high toxicity to honey bees, with an oral LD_{50} lower than 1 μg/bee (regulatory and evaluat). These findings confirm the direct exposure of honey bees to harmful pesticide residues that affect the colony health, as previously described by other studies (Johnson et al., 2010; Sanchez-Bayo et al., 2016). Most of the residues with a high toxicity to honey bees were insecticides; however, some of the detected fungicides also possess a low oral LD_{50}, such as azoxystrobin (25 μg/bee), dithianon (25 μg/bee) or

Table 1
Pesticide residues detected in APIStrip samples during 2020.

Pesticide name	Main use	Nunber of detections										Average conc. (ng/APIStrip)[a]	Authorised by the EC (expiration approval) (European Commission ())
		Total	Sampling site (CS)										
			1	2	3	4	5	6	7	8	9		
Boscalid	Fungicide	50	14	6	2	5		3	3	9	8	1.1	Yes
Azoxystrobin	Fungicide	41		3	2			8	16	12		6.6	Yes
Tebuconazole	Fungicide	28		3		2		6	2	6	9	1.7	Yes
Imidacloprid	Insecticide	24	5	2	1	1	3			7	5	1.2	No (2020)
Propiconazole	Fungicide	23			1		4	6	2	10		0.9	No (2014)
Mandipropamid	Fungicide	19							7	12		3.0	Yes
Carbendazim	Fungicide, Metabolite	18								9	9	0.5	No (2014)
Fluopyram	Fungicide	18		8		2	4			1	3	1.1	Yes
Pyraclostrobin	Fungicide	16								4	12	1.6	Yes
Demeton-S-methylsulfoxide	Insecticide	13					7	6				1.1	No (2007)
Chlorpyrifos	Insecticide	12	3		3	3					3	14	No (2020)
DEET	Repellent	12	3		4	3		1			1	9.7	No[b]
Cymoxanil	Fungicide	11							6	5		0.9	Yes
Diazinon	Insecticide, Acaricide, Repellent	11	3		1	3			2	2		1.4	No (2007)
Permethrin	Insecticide	11			2	1	1	2		5		2.5	No (2000)
Prothioconazole-desthio	Metabolite	11					3	1	4	3		< LOQ	Yes
Thiacloprid	Insecticide	9					1	6	2			2.3	Yes
Deltamethrin	Insecticide	8					8					6.1	Yes
Chlorantraniliprole	Insecticide	7	1	1	2	3						1.1	Yes
Coumaphos	Acaricide	7								7		0.6	No[b]
Diuron	Herbicide	7						2	1	4		1.3	No (2018)
Fenuron	Herbicide	7						3	4			0.5	No
Metobromuron	Herbicide	7					2		5			1.4	Yes
Cypermethrin	Insecticide	6	2		1			1			2	179	Yes
Thymol	Acaricide, Fungicide	6		6								>1000	Yes
Ethiofencarb	Insecticide	5	2			3						22	No (2002)
Fipronil-sulfone	Metabolite	5	1	1		1				1	1	< LOQ	No[b]
HCB	Fungicide	5	2		3							1.9	No
Dithianon	Fungicide	4					2		2			2.8	Yes
Chlorpropham	Herbicide	3				3						0.5	No (2015)
Epoxiconazole	Fungicide	3								3		0.6	No (2020)
Indoxacarb	Insecticide	3					3					0.6	Yes
Metrafenone	Fungicide	3			1				1	1		0.5	Yes
Pymetrozine	Insecticide	3					1		1		1	3.4	No (2015)
Spirotetramat	Insecticide	3					1			2		0.8	Yes
Trichlorfon	Insecticide	3					3					0.6	No (2007)
Triflumuron	Insecticide	3			2					1		2.8	Yes
2,4′-DDE	Metabolite	2			2							< LOQ	No[b]
4,4′-DDD	Metabolite	2		1	1							< LOQ	No[b]
Chlorothalonil	Fungicide	2							1	1		< LOQ	No (2016)
Fenamidone	Fungicide	2			1					1		6.3	No (2018)
Fipronil	Insecticide	2					1				1	5.7	No (2017)
Fludioxonil	Fungicide	2	1			1						7.8	Yes
Fluxapyroxad	Fungicide	2			1					1		4.5	Yes
Omethoate	Insecticide, Acaricide	2								2		< LOQ	No
Oxasulfuron	Herbicide	2					2					0.5	No (2018)
Propamocarb	Fungicide	2							2			0.7	Yes
Prosulfocarb	Herbicide	2			1					1		0.8	Yes
Pyridalyl	Insecticide	2								2		1.9	Yes
Quinoxyfen	Fungicide	2					1		1			7.7	No (2014)
Tebufenozide	Insecticide	2					1	1				2.9	Yes
Teflubenzuron	Insecticide	2			1					1		0.6	No (2019)
Thiabendazole	Fungicide	2								1	1	1.8	Yes
4,4′-DDE	Metabolite	1			1							< LOQ	No[b]
4,4′-DDT	Insecticide	1									1	0.5	No (1972)
Acetamiprid	Insecticide	1							1			1.5	Yes
Alachlor	Herbicide	1								1		< LOQ	No (2006)
Ametoctradin	Fungicide	1									1	< LOQ	Yes
Chlorfenvinphos	Insecticide, Acaricide	1						1				1.2	No
Dichlofluanid	Fungicide	1	1									< LOQ	No (2002)
Difenoconazole	Fungicide	1				1						1.9	Yes
Flubendiamide	Insecticide	1			1							0.7	Yes
Flucythrinate	Insecticide, Acaricide	1	1									1.2	No (2002)
Fluquinconazole	Fungicide	1					1					< LOQ	Yes
Hexaconazole	Fungicide	1				1						< LOQ	No (2006)
Hexythiazox	Acaricide	1									1	< LOQ	Yes
Isopyrazam	Fungicide	1			1							0.5	Yes
Metolachlor	Herbicide	1	1									2.1	No (2002)

(*continued on next page*)

Table 1 (*continued*)

Pesticide name	Main use	Nunber of detections									Average conc. (ng/APIStrip)[a]	Authorised by the EC (expiration approval) (European Commission ())
		Total	Sampling site (CS)									
			1	2	3	4	5	6	7	8	9	
Ofurace	Fungicide	1				1						3.3	No (2002)
Penthiopyrad	Fungicide	1			1							< LOQ	Yes
Propyzamide	Herbicide	1								1		< LOQ	Yes
Spiroxamine	Fungicide	1			1							3.2	Yes
Tetraconazole	Fungicide	1								1		< LOQ	Yes
Tricyclazole	Fungicide	1			1							0.6	No (2016)
Trifloxystrobin	Fungicide	1				1						1.2	Yes

[a] < LOQ represents that the pesticide was detected and identified at a concentration below the instrumental limit of quantification (0.5 ng/APIStrip).
[b] Never notified and authorised in the EU for its use in agriculture, as stated by the EC (European Commission ()).

chlorothalonil (40 μg/bee) (regulatory and evaluat). Pesticides with herbicide properties, such as diuron or fenuron, were also detected.

The average concentrations were in most cases lower than 5 ng/APIStrip, although these values are relative to the passive sampler and cannot yet be related to findings in other apicultural samples, nor to toxicity effects on the honey bee health. Nevertheless, they are comparable among them, and can be employed to assess which pesticides are present inside the beehives at a higher rate. For instance, it can be seen that cypermethrin showed the highest average concentration in its six detections in APIStrip samples (179 ng/APIStrip), followed by ethiofencarb and chlorpyrifos (22 and 13 ng/APIStrip, respectively).

Approximately half of the pesticides shown in Table 1 are currently approved by the EC for their use in agricultural crops. The detection of pesticides which are not currently approved is due, in most cases, to a) the persistence in the environment of some of these substances, b) a recent withdrawal for their approval, c) legal non-agricultural applications of these compounds (such as gardening or road shoulders), or d) and illegal agricultural use. DDT and its derivatives (DDE and DDD) constitute the most representative example of very persistent compounds that were banned long ago but that are still found: their half-life period has been estimated to last up to 6200 days (DT_{50} in soil) (regulatory and evaluat). Other pesticide residues exhibit a faster degradation in the environment but have been recently banned (such as imidacloprid, banned in 2020 and detected in 13% of samples). Examples of compounds with alternative uses include coumaphos -an acaricide typically employed in apiculture-, or fipronil -a veterinary treatment-, among others. Concretely, coumaphos was detected at low concentration levels in 7 APIStrips (4% samples), although it was not applied by the beekeepers that took part in the study. Its presence is probably due to the cross-contamination from surrounding apiaries or its migration from recycled beeswax, as reported in previous studies (Murcia-Morales et al., 2021).

3.1.2. *Pesticide distribution over the sampling sites*

A substantial variation in the findings of the nine sampling sites was detected (Fig. 2a and Table 1). Apiary 8, located in the northeast of the Central Denmark Region, was the place with the largest number of detections and pesticide diversity –i.e. 117 detections and 31 different pesticide residues. However, the average concentration of contaminants in this apiary was the smallest: 1.3 ng/APIStrip. By contrast, in apiary 9 (northwest of the Central Denmark Region), the average concentration of pesticides was 39.6 ng/APIStrips, partly due to the contribution of cypermethrin. APIStrips placed in apiary 3 showed also a high average concentration in the detections (15.6 ng/APIStrip), together with a significant pesticide diversity (25 different pesticide residues identified).

Out of the 428 pesticide residues included in the study, boscalid was found to be the one with the widest distribution, with detections in eight of the nine sampling sites. The only place where it was not detected was sampling site 5, located in the North Jutland Region. Imidacloprid and tebuconazole residues were as well present in a high number of sampling sites, with detections in seven and six sampling sites, respectively. Other

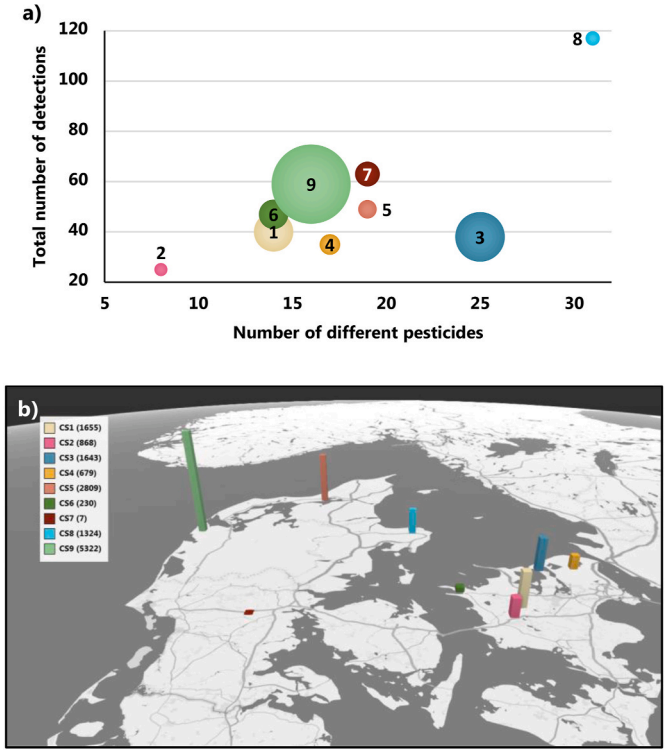


Fig. 2. a) Pesticide findings over the monitoring performed in 2020 in the nine sampling sites (CS1 to CS9, 20 APIStrips per sampling site); the size of the bubbles is proportional to the average concentration of the pesticides; b) APIStrip-Evaluated Toxicity for honey bees in each sampling site (the height of the bar illustrates the AET value).

seven pesticide residues were detected in more than half the sampling sites: DEET, azoxystrobin, permethrin, diazinon, propiconazole, fipronil-sulfone (metabolite of fipronil) and fluopyram, which were found in five sampling sites throughout the country. In some cases, a local application of a pesticide was seen, as in the case of chlorantraniliprole, which was identified in four sampling sites (1-2-3-4) located in a radius of approximately 25 km from each other. By contrast, 35 pesticide residues were detected uniquely in one sampling site.

Although the total pesticide load in the beehives is a useful indicator of the environmental contamination, it is not necessarily related to high toxicity levels for the honey bees themselves. Some pesticide residues possess a very low toxicity to honey bees, and their presence in beehives –even at high concentration levels– is unlikely to cause serious harm to the colony strength or health. However, even a small amount of an active substance with a high toxicity to honey bees (i.e. low LD_{50}) could severely affect the colony status. To assess the potential consequences of the findings in the APIStrips from each apiary, a combined risk indicator

parameter was calculated, the APIStrip-Evaluated Toxicity (AET), as the sum of the concentrations of each pesticide (in ng/APIStrip) divided by their corresponding oral LD_{50} (μg/bee) value:

$$AET = \frac{concentration_1}{LD_{50_1}} + \ldots + \frac{concentration_n}{LD_{50_n}}$$

A high AET value means, therefore, the presence of a highly toxic pesticide for honey bees and/or high concentration of pesticides in the beehive environment. This parameter was calculated for each one of the nine apiaries that took part in the samplings in 2020 (CS1 to CS9), as can be observed in Fig. 2b. CS9 was found to be the apiary with the highest AET (5322); this was, also, the apiary with the highest average concentration of pesticides, as shown in Fig. 2a. The main contributor to this large AET value was cypermethrin, an insecticide with oral LD_{50} of 0.17 μg/bee found at high concentrations in this apiary. However, whereas CS5 showed a low average concentration of pesticides, the AET in this apiary was found to be 2809 (the second highest). This is mainly due to the presence of deltamethrin, imidacloprid and fipronil, with LD_{50} values as low as 0.004 μg/bee. Although these pesticides were always detected at low concentration levels, their high toxicity resulted in an intense contribution to the AET. It is also remarkable that, despite the high number of different pesticide residues detected in CS7, any of them showed a significant toxicity to honey bees, which resulted in an extraordinarily low AET of 7.

The AET values should be considered as a relative worst-case scenario: due to the APIStrip capability to absorb pesticides and contaminants from a large number of bees, the actual exposure of an individual honey bee will probably be lower –i.e. not all bees will be directly exposed to all the pesticides captured by the APIStrips. However, the synergistic effects caused by the combination of different pesticides inside the beehive should also be considered, as demonstrated previously in other studies (Wade et al., 2019). As all of the colonies involved in the study showed a good strength and health state during the sampling period, it can be stated that the AET values presented here do not imply a direct risk to honey bee health.

3.1.3. Pesticide distribution over the seasons

Fig. 3 shows the distribution of the detections throughout the 10 sampling rounds, in terms of total detections (green, left bars) and diversity of the pesticide residues (orange, right bars). Each bar comprises the total findings in the 18 APIStrips (nine apiculturists with two beehives each) sampled per round. In terms of pesticide diversity, there was an average of 23 different residues detected in each sampling round, which represents a 14-day period. As can be observed, the number of detections varied significantly between sampling rounds 1–6 (April 17th to July 10th) and 7–10 (July 24th to September 4th). In the first period, the average of detections in each sampling round was 70, whereas in the second, the average of findings dropped to 32. The diversity of pesticides exhibits a similar trend, with up to 40 different residues detected in sampling round 6 in contrast to the 20 or fewer different contaminants identified in sampling rounds 7 to 10. Fig. 6 also shows the average number of honey bees in 18 reference beehives, as estimated by the Danish coordinator and the citizen scientists. The number of bees (i.e. the colony strength) was calculated as the number of frames full of bees, assuming that a 810-cm^2 frame completely filled can contain up to 1000 honey bees (1.24 bees per cm^2), and standardizing the frame size of each individual colony. It can be observed that, in general terms, the maximum number of adult bees coincided with the highest pesticide detections. This supports the idea that honey bee activity is the main source of contamination in the beehives.

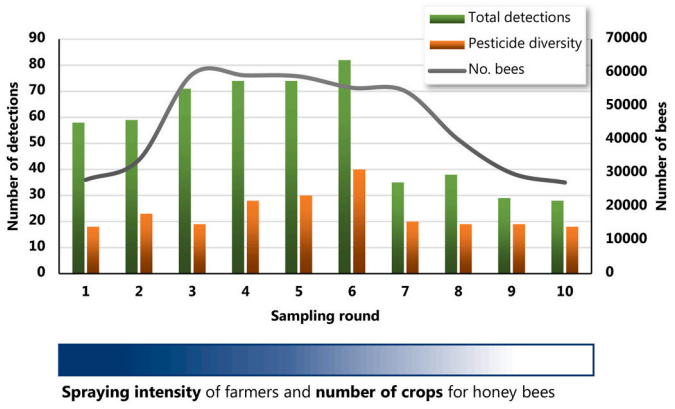


Fig. 3. Pesticide residue findings in 10 APIStrip-based sampling rounds over 2020 (April to September), and calculated number of bees in the colonies. Green bars (left) represent the total number of detections; orange bars (right) show the number of different pesticides identified. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The large numbers of detections during sampling rounds 1 and 2, even with a reduced number of honey bees in the colonies, could be explained on the basis of their activity. It has been previously reported that the foraging activity of honey bees is closely related to climate, being the most intense during the spring months and at temperatures close to 20 °C (Danner et al., 2017; Joshi and Joshi, 2010). Moreover, during the spring months, the number of farming crops and the pesticide application is typically very high, so honey bees are expected to be more exposed to these contaminants in this season. Afterwards, during the summer (intermediate sampling rounds), both the number of honey bees and pesticide findings in APIStrips are maximum. Earlier studies have shown that the greatest foraging activity of bees in Denmark takes place during the first half of July, which also supports the high number of detections found in the APIStrips from sampling rounds 5 and 6 (Poulsen, 2015).

The significant decrease in the detections after sampling round 6 (mid July 2020) might be related to a) the drastic decline in the number of honey bees in the colonies or b) a reduction in the number of farming crops and, therefore, the pesticide application (as shown in Fig. 3). The number of flowering agricultural crops that are of importance for honey bees decreases gradually through the summer and, also, the spraying of plant protection products is less intense after that period. This might result in a significant decrease of the pesticide presence in the environment.

Fig. 4 illustrates three different tendencies that were identified in the pesticides with a high number of detections. Whereas most of them, such a azoxystrobin, tebuconazole or thiacloprid (Fig. 4a) were detected most frequently during the spring months (first sampling periods) and their incidence in the environment decreased over time, the opposite happened with others such as pyraclostrobin or imidacloprid (Fig. 4c). The higher detections of the latter pesticide in the apiaries might be, among other factors, related to the decrease in the number of living bees over the last sampling rounds. There were also pesticides whose maximum presence in the environment was detected during the summer months (intermediate sampling periods), as shown in Fig. 4b. These tendencies can also be explained on the basis on the agricultural crops growing near the apiaries: the periods when a pesticide residue is more frequently detected might correspond to the application of this active substance in agricultural lands.

3.2. Monitoring 2019–2020

Four of the apiculturists involved in the samplings that took place in 2020 had already participated in a preliminary monitoring the previous year, whose results were already presented (Murcia Morales et al., 2020a,b). As the experimental conditions and sampling periods were the same in both years, the findings can be assessed as a two-year monitoring of the environment in the island of Zealand (sampling sites 1, 2, 3 and 4). The number of different pesticides –i.e. the pesticide diversity– remained virtually constant, with 39 and 38 pesticide residues detected

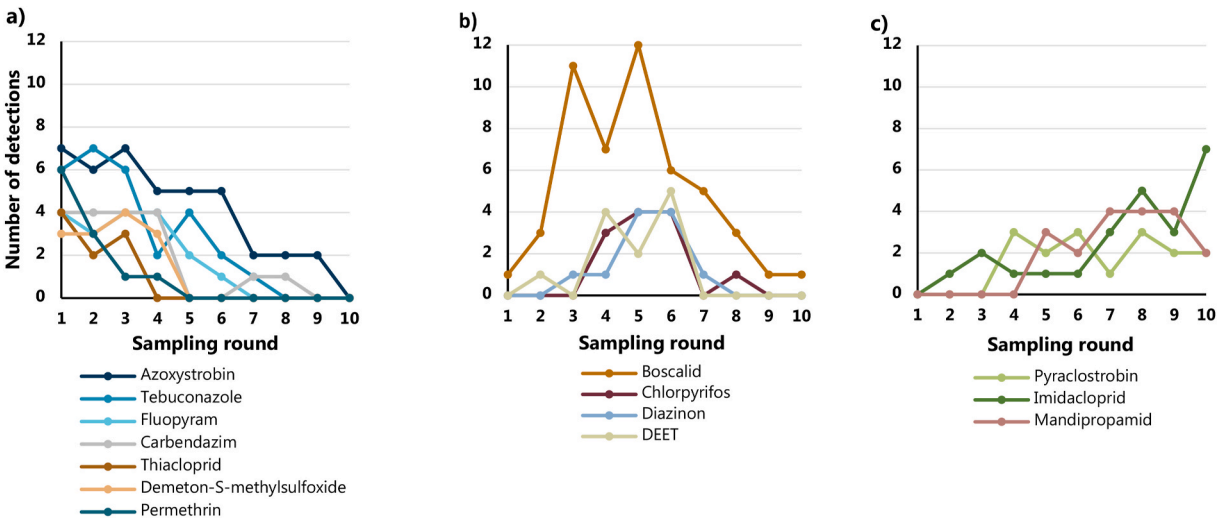


Fig. 4. Distribution over the seasons of selected pesticide residues detected over 2020. a) Pesticides with a high frequency of detection during the first sampling periods (spring); b) pesticides frequently detected on the intermediate sampling periods (beginning of summer); c) pesticides identified at a later stage of the samplings (end summer).

in 2019 and 2020, respectively. Fifteen pesticide residues (39% of the total detections) were identified in samples from both years, boscalid being always the most frequently detected residue. Fig. 5a depicts the 12 pesticide residues found in both 2019 and 2020 (sampling sites 1–4), in terms of the percentage of APIStrips where they were identified. It can be seen that, in general, the frequency of detection for these contaminants remained considerably constant throughout both years, the most notable exception being azoxystrobin. In this case, the compound was the second most frequent contaminant in 2019, but its incidence in APIStrips from 2020 was considerably lower. A reduction in its

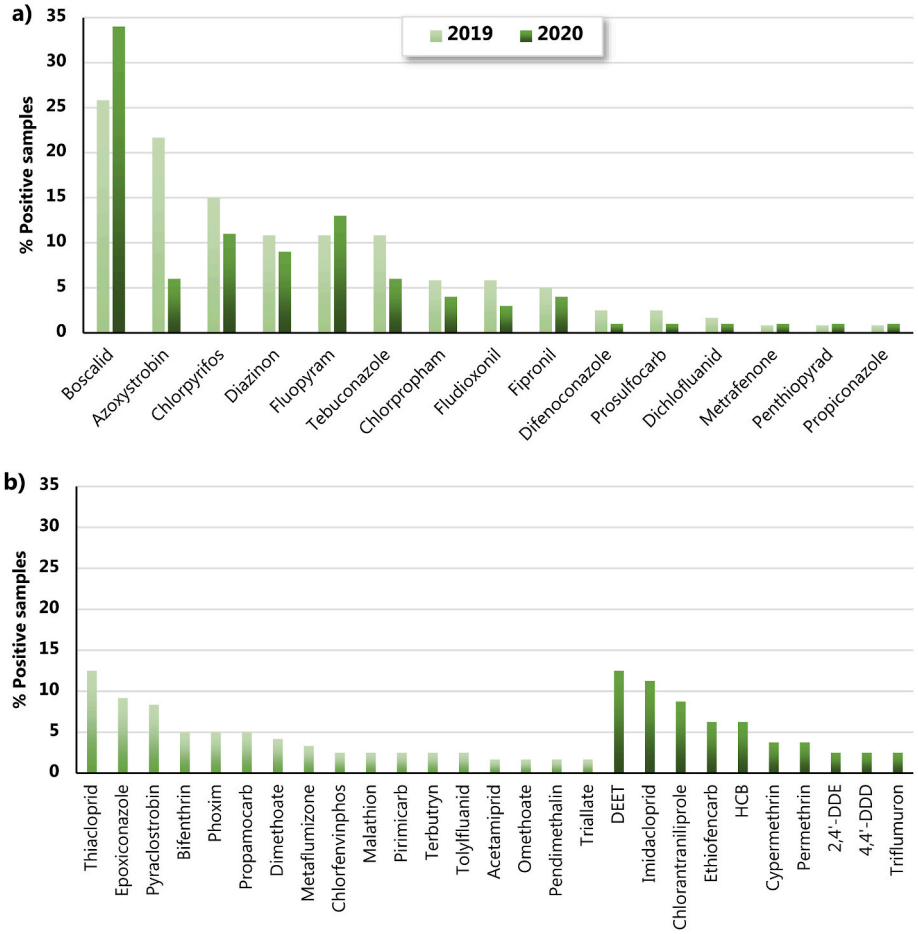


Fig. 5. Findings in the samplings performed in 2019 (pale green) and 2020 (dark green). a) Pesticides found throughout the two-year monitoring; b) pesticides detected only in one year. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

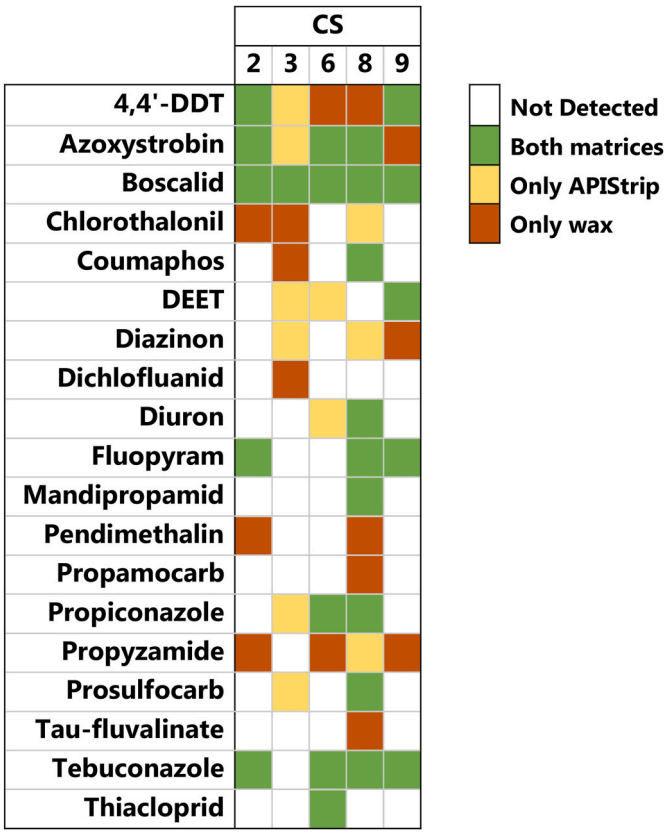


Fig. 6. Comparison between the pesticide residues detected in wax and APIStrip samples from CS2, CS3, CS6, CS8 and CS9.

agricultural application might have influenced this decrease: the sales of azoxystrobin in Denmark during 2018 (6140 kg) were significantly lower than in previous years (average 20043 kg sold annually in the period 2014–2017). This could be related to the weather conditions (the application of fungicides varies considerably depending on precipitation and temperature), or a tendency to substitute this fungicide (Miljøstyrelsen, 2019).

Conversely, Fig. 5b represents which pesticides were detected in only one of the sampling years (sampling sites 1–4). For simplicity reasons, the residues detected in only 1% of samples have not been included. It can be seen that none of the pesticides detected in only one year was present in more than 13% of samples, and the majority of them were detected in less than 5% of samples, which reveals a low incidence of these substances.

3.3. Wax and honey samples

Two wax samples (one per beehive) were taken from selected sites at the end of the sampling period in 2020: CS2, CS3, CS6, CS8 and CS9, resulting in 10 wax samples analyzed. Nineteen different pesticide residues were detected in these samples, with 40 total detections (Fig. 6). In the vast majority of cases, the concentrations levels ranged between 0.5 and 5 μg/kg, the exceptions being DEET and prosulfocarb (up to 36 μg/kg).

These results were qualitatively compared to the findings in APIStrip samples, as shown in Fig. 6: if a pesticide was detected in both the wax samples and the APIStrips from the same apiary, it appears in green colour. Conversely, brown colour depicts pesticides that were detected only in the wax samples (not in APIStrips), and yellow colour, a detection only in APIStrips. It can be seen that, in five compounds, there was a total equivalence in the detections: boscalid, fluopyram, mandipropamid, tebuconazole and thiacloprid, which were present in both matrices in all apiaries were they were found (only green colour). Others, such as DEET or propiconazole, were detected in APIStrips with a higher frequency than in wax (green and yellow colour). Conversely, out of the 19 pesticide residues detected in wax samples, four were not detected in APIStrip samples in these apiaries: dichlofluanid, pendimethalin, propamocarb and tau-fluvalinate. These compounds were detected in the wax samples at concentrations below 2 μg/kg. Their origin might not be environmental –they could be already present in the recycled wax foundation employed in these beehives. This is likely the case, especially, of tau-fluvalinate, which is a common miticide employed in apiculture with a very high persistence in wax (which would also explain why it did not migrate to the APIStrip) (Murcia-Morales et al., 2021).

In these five apiaries, the APIStrips detected a total of 51 different pesticide residues, 36 more than wax samples. These findings throughout the 6-month, APIStrip-based sampling should not be compared to one individual wax sampling, so they were not included in Fig. 6. However, such a long sampling period could not have been performed using the wax matrix: probably, the negative impact of 10 subsequent samplings on the colonies would have been too intense.

As regards the honey matrix, samples were taken from each apiary in two different occasions: one on the first half of the sampling period in 2020, and another on the second half. This resulted in 18 honey samples, in which only five different pesticide residues were detected: azoxystrobin (Maximum Residue Level –MRL– in honey 50 μg/kg), boscalid (MRL 150 μg/kg), fluopyram (MRL 50 μg/kg), tebuconazole (MRL 50 μg/kg) and thiacloprid (MRL 200 μg/kg) (regulatory and evaluat). Thiacloprid was the pesticide with the highest number of detections, being found in seven honey samples at concentrations levels up to 7.2 μg/kg. The remaining pesticides were detected in just one honey sample (except for azoxystrobin, with two detections) at concentrations below 1 μg/kg, resulting in 12 total detections for all compounds. The case of thiacloprid is of especial interest, as it is a neonicotinoid toxic to honey bees: it was found in honey from apiaries CS1, CS5, CS6 and CS8. For their part, APIStrips detected imidacloprid in CS5, CS6 and CS7. Thiacloprid is a polar compound (log P 1.26) which can easily accumulate in a hydrophilic matrix such as honey, which explains the large number of detections in these samples. In all cases, the maximum concentration detected of these pesticides was close to 100 times lower than their MRL in honey.

Conversely, in the 18 APIStrips that were taken simultaneously to the honey samples, 17 pesticide residues were detected (azoxystrobin, boscalid, carbendazim, cymoxanil, deltamethrin, fluopyram, imidacloprid, mandipropamid, metobromuron, oxasulfuron, permethrin, propamocarb, propiconazole, pyraclostrobin, quinoxyfen, spirotetramat and thrichlorfon). Therefore, the analysis of only honey samples would have reported less than one third of the possible detections with the use of APIStrips.

4. Conclusions

APIStrips have shown to be suitable for long-term, large-scale monitoring programs aimed at assessing the presence of contaminants in the environment. Their continuous use in beehives did not cause any observable harm in the honey bee colonies, while providing consistent and comprehensive information about the pesticides in surrounding areas. Boscalid was the most frequently detected compound in both 2019 and 2020, and also the one with the widest distribution throughout the country. During 2020, 75 different pesticide residues were detected, with significant variations in the nine apiaries used as sampling sites: the total number of detections ranged from 25 to 117, and the pesticide diversity (number of different pesticides found), from 8 to 31. The average concentration of pesticides was low, with few exceptions including cypermethrin. A relative indicator of the potential risk of these substances for the honey bee health was calculated, considering the LD_{50} and the concentration of each pesticide detected with the APIStrips;

however, further studies are needed to relate the indicator values to effects on the colony health. The pesticide load in the environment varied substantially in the different sampling rounds, both in terms of total detections (highest in mid July) and abundance of a certain pesticide. The results from both years were consistent to each other and to the sales of phytosanitary substances in Denmark. The analysis of wax and honey samples showed to be less representative and provide less information than the APIStrips for environmental monitorings.

Author statement

Maria Murcia-Morales: Methodology, Validation, Investigation, Writing - Original Draft, Visualization, Francisco José Díaz-Galiano: Methodology, Formal analysis, Investigation, Writing - Review & Editing, Flemming Vejsnæs: Methodology, Investigation, Resources, Writing - Review & Editing, Supervision, Ole Kilpinen: Methodology, Investigation, Resources, Writing - Review & Editing, Jozef J. M., Van der Steen: Conceptualization, Resources, Writing - Review & Editing, Project administration, Funding acquisition, Amadeo R. Fernández-Alba: Conceptualization, Writing - Review & Editing, Visualization, Supervision.

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.

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