Charakterisierung der Qualität von Blütenpollen in unterschiedlichen Regionen Baden-Württembergs

Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)

Fakultät Naturwissenschaften

Universität Hohenheim

vorgelegt von

Carolin Gertrud Maria Friedle

aus *Stuttgart*

2022

Dekan der Fakultät Naturwissenschaften:	Prof. Dr. Uwe Beifuß
1. Prüfer:	Prof. Dr. Martin Hasselmann
2. Prüfer:	Prof. Dr. Jan Frank
3. Prüfer:	PD Dr. Peter Rosenkranz

Einreichung der schriftlichen Dissertation am: 10.11.2021

Datum der mündlichen Prüfung: 18.02.2022

Inhaltsverzeichnis

Erklärung des Autors

Die hier präsentierte Arbeit wurde unter der Betreuung von Prof. Dr. Martin Hasselmann und Prof. Dr. Walter Vetter an der Landesanstalt für Bienenkunde an der Universität Hohenheim von April 2018 bis November 2021 durchgeführt.

Der größte Anteil der Arbeit wurde bereits in internationalen „peer-review“ Journalen veröffentlicht bzw. ist noch im „peer-review“ Prozess:

Veröffentlichung 1: Friedle, C., Wallner, K., Rosenkranz, P., Martens, D., Vetter, W. (2021): Pesticide residues in daily bee pollen samples (April-July) from an intensive agricultural region in Southern Germany. In: *Environ Sci Pollut Res*, S. 1-15. DOI: 10.1007/s11356-020-12318-2.

Veröffentlichung 2: Friedle, C.; D'Alvise, P.; Schweikert, K.; Wallner, K.; Hasselmann, M. (2021): Changes of microorganism composition in fresh and stored bee pollen from Southern Germany. In: *Environ Sci Pollut Res*. DOI: 10.1007/s11356-021-13932-4.

Veröffentlichung 3: Friedle, C., Kapp, T., Wallner, K., Alkattea, R., Vetter, W. (2022): High abundance of pyrrolizidine alkaloids in bee pollen collected in July 2019 from Southern Germany. In: *Environ Moni and Ass.* DOI: 10.1007/s10661-022-09907-8.

Zusätzlich wurden Teile dieser Arbeit bei nationalen wissenschaftlichen Konferenzen in Posterbeiträgen präsentiert:

- 65. Tagung der AG der Institute für Bienenforschung e.V. in Koblenz, März 2018: „Entwicklung und Validierung eines schnellen Analyseverfahrens zur Messung von Pflanzenschutzmittelrückständen im Nektar heimkehrender Bienen mittels GC-ECD und LC-MS/MS“
- 47. Deutsche Lebensmittelchemiker Regionaltagung (GDCh) in Gießen, März 2018: „Entwicklung und Validierung eines schnellen Analyseverfahrens zur Messung von Pflanzenschutzmittelrückständen im Nektar heimkehrender Bienen mittels GC-ECD und LC-MS/MS“
- 66. Tagung der AG der Institute für Bienenforschung e.V. in Frankfurt, März 2019: „Charakterisierung der Qualität von Blütenpollen“

- 48. Deutsche Lebensmittelchemiker Regionaltagung (GDCh) in Dresden, September 2019: „Untersuchung der mikrobiellen Zusammensetzung von Blütenpollen“

Die Beteiligung und Beiträge aller Autoren, die an dieser Arbeit mitgewirkt haben, werden wie folgt erwähnt:

Carolin Friedle hat im Wesentlichen alle praktischen Arbeiten, mit Ausnahme der unten aufgeführten Arbeiten der Co-Autoren durchgeführt. Die durchgeführten Arbeiten umfassen das Konzipieren und die Organisation des Probensammelns der **Veröffentlichungen 1**, **2** und **3**, sowie die teilweise selbstständige Durchführung des Sammelns der Proben für **Veröffentlichung 2** und **3**. Darüber hinaus führte sie die Probenaufarbeitung mittels QuEChERS an der Landesanstalt für Bienenkunde in **Veröffentlichung 1** und mittels TRIzol Protokoll am Institut für Populationsgenomik in **Veröffentlichung 2** durch, sowie das Anfertigen der Pollenpräparate an der Landesanstalt für Bienenkunde zur palynologischen Analyse in **Veröffentlichung 1**, **2** und **3**. Zudem führte sie die gesamte Dateninterpretation durch und erstellte alle Manuskripte einschließlich der Abbildungen und Tabellen. Sie war auch für alle formalen Aspekte des Veröffentlichungsprozesses aller Veröffentlichungen verantwortlich.

Prof. Dr. Martin Hasselmann hat die vorliegende Arbeit betreut und sowohl bei der Konzeption der Versuchsdurchführung in **Veröffentlichung 2** als auch bei der Erstellung der **Veröffentlichung 3** unterstützt und Korrektur gelesen.

Prof. Dr. Walter Vetter hat die vorliegende Arbeit mitbetreut und sowohl bei der Konzeption der Versuchsdurchführung in **Veröffentlichungen 1** und **3** mitgewirkt als auch bei der Erstellung dieser Manuskripte unterstützt und Korrektur gelesen.

PD. Dr. Peter Rosenkranz hat die Durchführung der Versuche in **Veröffentlichung 1** und **3** mitkonzipiert, betreut und Korrektur gelesen.

Erklärung des Autors

Dr. Klaus Wallner hat innerhalb des Projekts die finanzielle Unterstützung beantragt und die praktischen Arbeiten an der Landesanstalt für Bienenkunde in **Veröffentlichung 1** und **3** betreut, das Sammeln der Proben aller Veröffentlichungen mitkonzipiert und Korrektur gelesen.

Dr. Paul D'Alvise hat die Aufarbeitung der Proben mittels TRIzol Protokoll und die Auswertung der Ergebnisse in **Veröffentlichung 2** betreut und diese ebenfalls Korrektur gelesen.

Dr. Raghdan Alkattea hat die palynologische Analyse in **Veröffentlichung 1**, **2** und **3** unterstützend durchgeführt, sowie **Veröffentlichung 3** Korrektur gelesen.

Dr. Dieter Martens hat die Analyse der Proben mittels LC-MS/MS an der Landwirtschaftlichen Untersuchungs- und Forschungsanstalt in Speyer (LUFA) in **Veröffentlichung 1** durchgeführt, sowie Korrektur gelesen.

Dr. Karsten Schweikert hat die statistische Auswertung in **Veröffentlichung 2** durchgeführt, sowie ein Teil der Abbildungen erstellt und Korrektur gelesen.

Thomas Kapp hat die Analyse mittels Festphasenextraktion und Messung der Proben mittels LC-MS/MS am Chemischen und Veterinäruntersuchungsamt (CVUA) in Stuttgart in **Veröffentlichung 3** durchgeführt und Korrektur gelesen.

Mit der Hilfe von freiwilligen Imker*innen in Baden-Württemberg wurden die Proben in **Veröffentlichung 1**, **2** und **3** gesammelt.

Fellbach, 06.03.2022	C. Friedle	
Ort und Datum	Unterschrift Autor	Unterschrift Betreuer
	Carolin Friedle	Prof. Martin Hasselmann

1 Allgemeiner und theoretischer Teil

1.1 Bienen und ihre Rolle als Bestäuber

Weltweit werden derzeit über 80% aller Wild- und Kulturpflanzen von Insekten bestäubt (Reddy et al. 2012; Bartomeus et al. 2014). Damit leisten Insekten nicht nur einen unersetzlichen Beitrag zur Aufrechterhaltung der Ökosysteme, sondern sorgen auch für die Sicherstellung der Nahrungsmittelversorgung des Menschen (Klein et al. 2007). Ohne den Beitrag von Bestäubern in der Nahrungsmittelherstellung wäre das Angebot von Lebensmitteln deutlich reduziert, viele Früchte, wie Stein- und Kernobst, Beeren, Gemüse oder Kaffee und Tee würden für den menschlichen Verzehr nicht mehr verfügbar sein (Steffan-Dewenter et al. 2005). Nicht nur der Mensch profitiert von der Bestäubung der Pflanzen und den daraus resultierenden Lebensmitteln, sondern auch Bestäuber und Pollen produzierende Pflanzen leben in einer gegenseitig profitierenden Wechselwirkung (Darwin 1859).

Neben Käfern, Schmetterlingen und Ameisen, tragen Bienen den größten Anteil zur Bestäubung von Pflanzen bei (McGregor 1976). Die westliche Honigbiene (*Apis mellifera*) zählt zu den häufigsten Blütenbesuchern in natürlichen Lebensräumen weltweit (Hung et al. 2018), denn sie wird als Nutztier zur Honigproduktion eingesetzt. Ein über 100 Millionen Jahre alter fossiler Fund einer männlichen Biene (*Melittopshex brumensis*) mit Pollenkörnern an den Beinen beweist, dass eine evolutionäre Verknüpfung von Angiospermen (bedecktsamigen Pflanzen) und Bestäubern schon seit Millionen Jahren besteht und Bienen schon damals zur Bestäubung beigetragen haben (Poinar und Danforth 2006; Cardinal and Danforth 2013).

Honigbienen (*Apis mellifera*) leben in hochsozial organisierten Insektenstaaten mit mehr als 30.000 Bienen (van der Steen 2015). Die Aufgaben der Arbeiterinnen werden nach ihrem Lebensalter bestimmt. In den ersten drei Tagen nach dem Schlüpfen sind die Jungbienen für das Säubern der Zellen verantwortlich, danach folgt die Versorgung der Larven, die Bildung von Wachs und der Bau von Waben. Ab dem 21. Tag beginnen Honigbienen mit dem Ausfliegen

aus dem Stock, um Nektar, Pollen, Propolis und Wasser zu sammeln (Ohe 2006). Der Nektar von blühenden Pflanzen dient als Energiequelle der Honigbienen und wird zur Produktion von Honig gesammelt. Die Hauptproteinquelle der Bienen ist Blütenpollen, der zur Versorgung der Brut und Produktion von Futterdrüsensaft verwendet wird (Winston 1987; Seeley 1997). Pflanzen die durch Tiere bestäubt werden, bieten diesen meist neben Pollen auch Nektar an, so werden mehrere Blütenbesuche der gleichen Pflanzenspezies und die Verteilung des produzierten Pollens sichergestellt (Free 1963).

Honigbienen sind blütenstetig und fliegen bei ihren Sammelflügen meist nur Blüten von Pflanzen der gleichen Art an, dabei bleiben die Pollenkörner am Haarkleid der Bienen hängen und werden von Blüte zu Blüte getragen (Kevan und Baker 1983). Im Gegensatz zu nacktsamigen Pflanzen (*Gymnospermae*), die hauptsächlich windbestäubt werden, sind bedecktsamige Pflanzen (*Angiospermae*) abhängig von der Bestäubung durch Insekten (entomophil) (s. Abbildung 1).

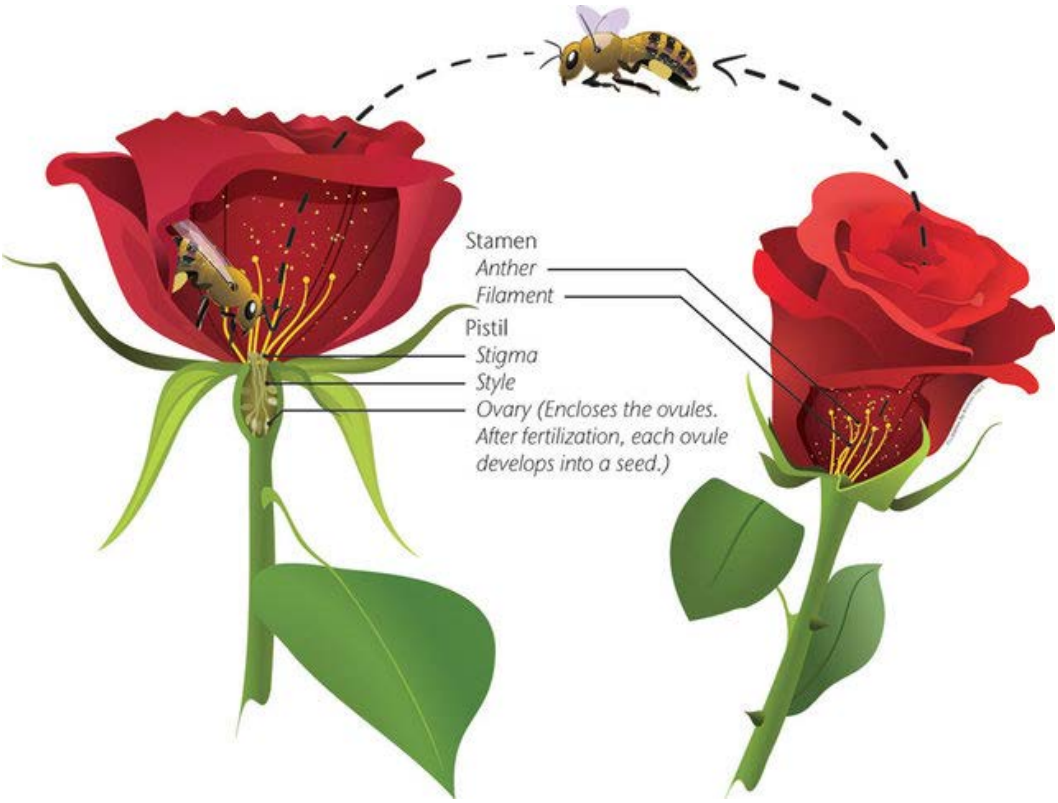

Abbildung 1: Entomophile Pflanzen, die durch Blütenbesuche bestäubt werden (Lawrence 2015).

Im Flug streifen die Bienen mit den Bürsten an ihren Beinpaaren die Pollen zum hinteren Beinpaar (Willmer 2011). Durch Zugabe von Speichelsekret und Nektar werden kleine Klümpchen geformt und am hinteren Beinpaar befestigt (Winston 1987). Als sogenanntes Pollenhöschen (ca. 10 mg), das aus mehreren Milliarden einzelner Pollenkörner besteht, transportieren Honigbienen die Pollen am hinteren Beinpaar in den Stock (Winston 1987; Ohe 2004). Unter weiterer Zugabe von Bienenspeichel werden die Pollen in Waben eingelagert und zu sogenanntem Bienenbrot fermentiert (Nagai et al. 2005). Das Bienenbrot wird von Jungbienen und den Arbeiterinnen im Stock genutzt, um Larvenfuttersaft zur Aufzucht der Brut zu produzieren (Lindauer 1952). Ein Bienenvolk benötigt im Durchschnitt 20 kg Pollen pro Jahr, der vollständig zur Versorgung der Brut genutzt wird (Crailsheim et al. 1992).

Um die Versorgung des Bienenstocks mit Futter über das gesamte Bienenjahr gewährleisten zu können, beginnen Bienen zu Beginn des Frühjahres (März) meist mit dem Sammeln von Weidenpollen (*Salix sp.*), sobald die Königin mit dem Legen von Eiern (stiften) beginnt (Winston 1987; Harz M. 2014). Beendet wird das Sammeln von Pollen erst im Herbst (September/Oktober), sobald das Trachtangebot nachlässt und die Königin das Stiften reduziert (Throp 2000). Je nach Trachtangebot und -attraktivität kann der Sammelradius von Honigbienen zwischen 1.500 m und 10.000 m betragen (Beekman und Ratnieks 2000; Steffan-Dewenter und Kuhn 2003). Attraktive Pflanzen für Honigbienen können Wildpflanzen wie Kräuter oder Wildblumen, als auch kultivierte Pflanzen wie Raps oder Obstbäume sein (McGregor 1976).

1.2 Pollen

Pollenkörner oder auch Blütenstaub genannt, sind die männlichen Keimzellen (Mikrosporen) der Blütenpflanze (Cruden 1999). Sie besitzen eine doppelte Außenschicht (Sporoderm), die durch eine innere Zelluloseschicht (Intine) und eine widerstandsfähige äußere Schicht (Exine)

mit einer artspezifischen Oberflächenstruktur, gekennzeichnet ist (Piffanelli et al. 1998). Die Vielfalt der Pollenkorntypen ist vor allem bei Bedecktsamern durch Anpassung an die Tier-Bestäubung sehr ausgeprägt und systematisch bedeutungsvoll (Piffanelli et al. 1998). Eine unregelmäßige Oberflächenstruktur der Pollen begünstigt das Anhaften im Haarkleid von Insekten und die Bestäubung anderer Pflanzen (Piffanelli et al. 1998). Die Größe der Pollenkörner kann je nach Pflanzengattung zwischen 5 - 200 µm variieren, dabei sind Form und Oberflächenstruktur sind sehr vielfältig (Ohe und Ohe 2007) (s. Abbildung 2). Eine Identifizierung und Unterscheidung der Pollenkörner verschiedener Pflanzengattungen ist bislang unter dem Mikroskop mittels palynologischer Analyse möglich (s. Kapitel 1.5.1).

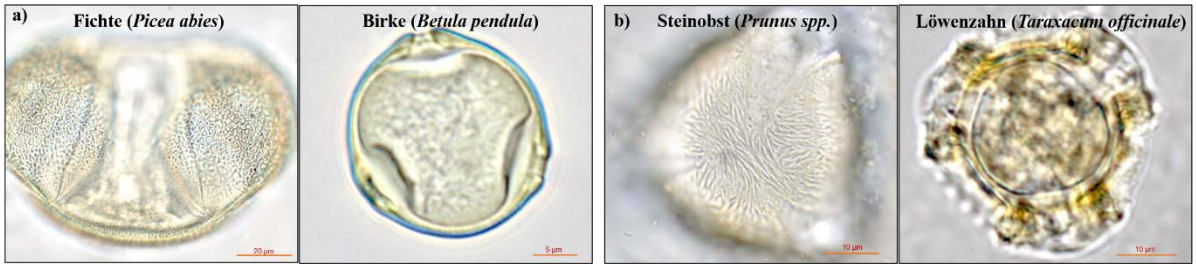

Abbildung 2: Pollenkörner von a) nacktsamigen und b) bedecktsamigen Pflanzen

1.3 Pollen als Lebensmittel

Pollen haben einen hohen ernährungsphysiologischen Wert, denn sie enthalten einen großen Anteil an Proteinen zwischen 11 und 35% (Haydak 1970). Neben Proteinen sind auch weitere ernährungsrelevante Nährstoffe wie essentielle Aminosäuren, Fettsäuren, Vitamine, Mineralstoffe und Enzyme in Pollen zu finden (Basim et al. 2006; Morais et al. 2011; Feás et al. 2012; Avni et al. 2014; Pascoal et al. 2014; Kostić et al. 2015; Thakur und Nanda 2020; Campos et al. 2010) (s. Tabelle 1).

Tabelle 1: Inhaltsstoffe in Bütenpollen.

Inhaltsstoffe	Anteil in Blütenpollen
Wasser	20-30%
Proteine (essentielle Aminosäuren)	11 - 35%
Fette/Fettsäuren	1 - 22%
Kohlenhydrate	20 - 48%
Mineralstoffe	1 - 8%
Vitamine: Provitamine A, B_1, B_2, B_6, C, D, E, H, K, Biotin, Folsäure	
Enzyme, antibiotische Stoffe, Aroma und Farbstoffe	

Die unterschiedlichen Nährstoffe des Pollens sind nicht nur für Bienen essenziell, sondern auch für den menschlichen Verzehr interessant (Carpenter 2003). In der Imkerei werden deshalb neben Honig auch andere Bienenprodukte wie der sogenannte „Blütenpollen“ gewonnen, der seit Jahrtausenden von Menschen verzehrt wird (Campos et al. 1997). Eine tägliche Menge von bis zu 15 g Blütenpollen, deckt den täglichen Bedarf an Aminosäuren, die ein Mensch benötigt (Peris 1984). Der Begriff „Blütenpollen“ bezeichnet die Pollenhöschen der Sammlerbienen, die vor dem Transport in den Stock vom Imker abgesammelt werden. Mit Hilfe von sogenannten Pollenfallen können zwischen 10 - 40% der transportierten Pollenhöschen der Sammlerinnen abgefangen werden (Keller et al. 2005). Ein integriertes Gitter sorgt dafür, dass die Pollenhöschen abgestreift werden und aus einer darunter liegenden Schublade geerntet werden können. Die Effektivität der Pollenfallen ist abhängig von der Maschenweite des Gitters, die optimal bei 4 - 6 mm liegt (Seeley 1997). Es gibt unterschiedliche Arten von Pollenfallen, solche die vor das Flugloch installiert werden (s. Abbildung 2) und welche die im Beutenboden integriert werden, sie sind nicht standardisiert und variieren je nach Beutentyp in Form und Material (Detroy et al. 1976) .

Abbildung 3: Aufbau einer installierten Pollenfalle vor dem Flugloch eines Bienenstocks

Die Sammelperiode von Blütenpollen ist abhängig von der Region und des Standortes der Bienenbeuten (Keller et al. 2005). In Deutschland wird Blütenpollen üblicherweise zwischen März und September gesammelt (Keller et al. 2005). Das tägliche Leeren und Reinigen der Falle ist dabei unerlässlich, um ein mögliches mikrobielles Wachstum zu verhindern (De-Melo et al. 2016). Die Tagesernte aus der Pollenfalle kann je nach Wetter und Trachtangebot variieren und muss zur Gewährleistung der Haltbarkeit anschließend verarbeitet werden (González et al. 2005). Jährlich kann von einem Bienenvolk zwischen 1,2 – 50,65 kg Pollen geerntet werden (Keller et al. 2005; Campos et al. 2010), jedoch sollte das Sammeln von Blütenpollen regelmäßig pausiert werden, um die Gesundheit des Bienenvolkes über das Jahr gewährleisten zu können (Wilde 1991).

1.4 Qualität von Pollen

1.4.1 Einfluss von Pflanzenschutzmitteln

Äußere Einflüsse können die Qualität des Blütenpollens maßgeblich beeinflussen (De-Melo et al. 2015; Roßberg und Harzer 2015). Der Einsatz von Pflanzenschutzmitteln (PSM) in der

Landwirtschaft, kann zu Rückstandsbelastungen in der Umwelt, sowie in Trinkwasser und Nahrungsmitteln führen (Müller et al. 2002; Codling et al. 2016; Tadeo 2019). PSM können gezielt in blühende Kulturen eingesetzt werden, um gegen Krankheiten und Schädlinge wie beispielsweise Schorf und Mehltau im Obstbau oder den Rapsglanzkäfer und Rapsschwärze im Raps (*Alternaria brassicae*) zu bekämpfen (LTZ 2021a, 2021b). Folglich können PSM durch direkte Spritzmaßnahmen oder Behandlung von Unterwuchs in die Umwelt abdriften (Aktar et al. 2009). Demnach können PSM mit Nicht-Ziel-Organismen wie Insekten in Kontakt kommen (Koch und Weißer 1997). Die Bienenschutzverordnung regelt, wie mit bienengefährlichen Stoffen umgegangen wird. Demnach dürfen Wirkstoffe, die nach Bienenschutzverordnung als bienengefährlich (B1) eingestuft sind, nicht in blühende Kulturen ausgebracht und Wirkstoffe die minder bienengefährlich (B2) eingestuft sind erst nach Beflug der Blüte (nachts) eingesetzt werden (BienSchV 1992). Doch auch durch den Einsatz von Wirkstoffen in nicht blühende Kulturen können Bienen und andere Insekten durch Abdrift, beispielsweise durch blühenden Unterwuchs bzw. Nachbarkulturen, von Sprühapplikationen getroffen werden (Pimentel 1995; Botías et al. 2016).

In Deutschland sind derzeit mehr als 1780 PSM mit verschiedenen Handelsnamen und über 280 unterschiedlichen Wirkstoffen zur Anwendung zugelassen (BVL 2021b). Der Inlandsabsatz lag 2019 bei 27.000 Tonnen Wirkstoff, die Gruppe der Herbizide machte mit 50,6% den größten Anteil der abgegebenen PSM aus (Umweltbundesamt 2020). Neben Herbiziden kommen auch Fungizide und Insektizide sowohl in der Landwirtschaft, als auch zur Nutzung in Haus und Kleingärten zum Einsatz (BVL 2021b). Gerade B1 und B2 eingestufte Insektizide, die zur Bekämpfung von Schädlingen in der Landwirtschaft eingesetzt werden, können bei Fehlbehandlungen für Bestäuber gravierende Folgen haben (Czoppelt 1991; Tapparo et al. 2012). Bisherige Studien haben gezeigt, dass Honigbienen durch das Sammeln von Nektar und Pollen in bewirtschafteten Regionen unterschiedlichen PSM mit durchaus hohen Konzentrationen ausgesetzt

sind (Mullin et al. 2010; Stoner und Eitzer 2013; Böhme et al. 2018). Dabei wurden Auswirkungen von unterschiedlichen Wirkstoffen auf Honigbienen untersucht, die zeigen, dass die Entwicklung, die Orientierung, das Wachstum und die Überlebensfähigkeit stark beeinträchtigt werden können (Czoppelt 1991; Beliën et al. 2009). Auch additive oder synergetische Effekte durch die Kombination von Insektiziden aus der Gruppe der Pyrethroiden und Neonicotinoiden mit Azolfungiziden in Tankmischungen wurden belegt (Pilling et al. 1995; Gill et al. 2012; Thompson et al. 2014; Wernecke et al. 2019). Mögliche Gefahren, die durch PSM Rückstände in belasteten Pollen für Honigbienen entstehen, wurden in einzelnen Studien durch einen sogenannten Pollen Hazard Quotient (PHQ) berechnet (Stoner und Eitzer 2013; Böhme et al. 2018). Die Ergebnisse wurden allerdings auf Grundlage von wöchentlichen oder monatlichen Mischproben in bewirtschafteten Regionen und Naturräumen kalkuliert. Fundiertes Wissen über die tägliche Belastung von Pollen konnte bisher nicht erlangt werden. Ferner ist unklar, welche Wirkstoffkonzentrationen in täglich gesammelten Pollenproben aus intensiv bewirtschafteten Regionen erwartet werden kann, bzw. wie lange PSM nach der Applikation in Pollenproben detektierbar sind und welche Gefahr belastete Tagesproben für Honigbienen darstellen.

1.4.2 Einfluss von Mikroorganismen

Einen weiteren maßgeblichen Einfluss auf die Qualität von Blütenpollen haben Mikroorganismen (MO) (Dinkov 2018; Disayathanoowat et al. 2020). Durch einen relativ hohen Wasser- und Proteingehalt (s. Tabelle 1) bieten Blütenpollen als Rohprodukt Mikroorganismen viele Angriffspunkte (Feás et al. 2012). Mikroorganismen sind ubiquitär in der Umwelt, in der Luft, sowie im Wasser und im Boden zu finden (Stotzky 1972; Zalar et al. 2007). Folglich sind bestimmte MO auch in Pflanzen und in deren Nektar und Pollen präsent (Fridman et al. 2012). Honigbienen sind ebenfalls Träger unterschiedlicher Darmbakterien, die durch Speichelaustausch oder -zufuhr beim Sammeln auf Pollen transferiert werden können (Kwong und Moran 2016). Frisch

gesammelter Pollen enthält demnach eine Vielzahl an unterschiedlichen MO, die in der Umwelt präsent sind oder durch Honigbienen übertragen werden können. Die Zusammensetzung der MO in Blütenpollen hängt stark von der geografischen und botanischen Herkunft sowie von Klimabedingungen, dem Sammelzeitraum und der Verarbeitung nach dem Ernten ab (Hani et al. 2012; Nogueira et al. 2012; De-Melo et al. 2016). Folgt keine Verarbeitung wie Trocknung oder Kühlung des Pollens, können sich lebensmittelverderbende oder Toxin-bildende MO vermehren (Hani et al. 2012; De-Melo et al. 2015). Einige Studien haben bereits gezeigt, dass bei fehlender Verarbeitung MO wachsen, die beispielsweise Mykotoxine bilden (González et al. 2005; Fatrcová-Šramková et al. 2016). Zusätzlich wurde die Zusammensetzung von frischen, getrockneten und gefrorenen Blütenpollen basierend auf kultivierungsabhängigen Methoden sowie auf kultivierungsunabhängigen Methoden wie der 16S-rRNA Amplikonsequenzierung untersucht (Feás et al. 2012; De-Melo et al. 2016; Dinkov 2018). Die Veränderung der Mikroorganismenzusammensetzung wurde bisher nur bei Bienenbrot, dass 72 Stunden im Bienenstock gelagert wurde, untersucht (Disayathanoowat et al. 2020). Informationen über die Veränderung der Zusammensetzung von Mikroorganismen in Blütenpollen, der außerhalb des Bienenstocks gelagert wird, fehlen jedoch bislang.

1.4.3 Einfluss von Pyrrolizidinalkaloiden

Neben äußeren Einflüssen wie Pestiziden und Mikroorganismen können natürliche Inhaltsstoffe der Pollen einen enormen Einfluss auf die Qualität des Endprodukts ausüben (BfR 2013). Sekundäre Pflanzenmetabolite wie Pyrrolizidinalkaliode (PA) werden von der Pflanze zum Schutz vor Verbiss durch Fressfeinde produziert (EFSA 2011, 2017). Sie bestehen aus einem 1-Azabicyclo-[3.3.0]-octan (Pyrrolizidin) Grundgerüst mit einer Hydroxymethyl-Gruppe an der C1-Position, sowie einem Hydroxyl-Substituenten an der C7-Position. Die resultierende Grundstruktur ist eine Necinbase (7-Hydroxy-1-hydroxymethylpyrrolizidine), welche in

Abhängigkeit von der Veresterung als Monoester, Diester oder zyklische Ester auftreten können. Bei korrespondierenden N-Oxiden (PANO) liegt der Stickstoff im Molekül oxidiert vor.

Derzeit sind über 600 verschiedene Strukturen von PA bekannt, welche in ca. 3% aller blühenden Pflanzen weltweit auftreten können (EFSA 2011). Als Hauptquellen gelten hauptsächlich Pflanzen der Familien *Asteraceae* (*Senecioeae* und *Eupatorieae*), *Boraginaceae* und *Fabaceae* (Hartmann und Toppel 1987). In Deutschland ist bereits bekannt, dass Pflanzen wie *Echium sp*. (Natternkopf), *Borago sp.* (Borretsch), *Senecio sp*. (Kreuzkräuter) und *Eupatorium sp*. (Wasserdost) PA enthalten (Ohe 2018). PA, die eine 1,2-Doppelbindung enthalten, entwickeln nach der Aufnahme in Tier und Mensch toxische Wirkungen (EFSA 2011). Schon geringe Gehalte an PA können chronische Erkrankungen, sowie Leberschädigungen auslösen (Kakar et al. 2010; Colegate et al. 2012). Einige Studien mit Tierversuchen belegen ebenfalls die Karzinogenität einzelner Stoffe (NCI 1978; Chan et al. 2003). In Nahrungsmitteln mit botanischem Hintergrund wie Kräuter und Tees konnten bereits bedeutende Mengen an PA gefunden werden (Bodi et al. 2014). Auch Bienenprodukte wie Honig und Blütenpollen weisen in Untersuchungen Gehalte an PA auf (Boppré et al. 2005; Gottschalk et al. 2018). Bislang wurden kommerziell erhältliche Pollenprodukte aus EU- und Nicht-EU Ländern, sowie wöchentlich am Bienenvolk gesammelte Pollenproben aus der Schweiz untersucht (Dübecke et al. 2011; Kempf et al. 2011; Kast et al. 2018). Die Ergebnisse zeigen, dass vorwiegend PA die in Pflanzen wie Natternkopf (*Echium sp*.) und Wasserdost (*Eupatorium sp*.) vorkommen, nachgewiesen werden können. Kommerzielle Proben aus Deutschland sind bereits untersucht worden (Kempf et al. 2010a), jedoch ist bislang wenig über PA Gehalte in saisonal gesammelten Blütenpollen zu definierten Blühzeiten bekannt.

1.5 Analytik von Pollen

1.5.1 Mikroskopie

Pollenkörner können mittels palynologischer Analyse unter dem Mikroskop bestimmt werden (Klumpp 2013). Mittels dieser Analyse kann sowohl die botanische als auch die geographische Herkunft von Pollenkörnern bestimmt und dadurch Rückschlüsse gezogen werden, welche Blüten die Bienen angeflogen und wo sie Pollen gesammelt haben (Klumpp 2013; Disayathanoowat et al. 2020). Dazu wird der gesammelte Blütenpollen beispielsweise durch die Verwendung eines Mörsers homogenisiert und anschließend eine definierte Menge (beispielsweise 100 mg) in ein Probengefäß eingewogen. Durch Zugabe von Wasser und eines Tropfens Spülmittels, der dafür sorgt, dass sich Fetttröpfchen von den Pollenkörnen lösen, werden die Pollenkörner gelöst und durch Schütteln weiter homogenisiert. Eine definierte Menge (Bsp. 200 µL) wird auf einen Objektträgerglas gegeben und mit Glycerin-Gelatine und einem Deckglas bedeckt, sobald der Wassertropfen getrocknet ist. Unter dem Mikroskop (10x40 Vergrößerung) wird dann eine definierte Anzahl an Pollenkörnern (zwischen 300 und 500) ausgezählt und bestimmten Pflanzen zugeordnet (Friedle et al. 2021b; Friedle et al. 2021a).

Die Pollenkörner einiger Pflanzengenera/-arten weisen eine sehr spezifische Form und Größe auf und sind folglich unverwechselbar, andere zeigen sowohl in der Form als auch in der Größe Ähnlichkeiten zu Pollen andere Pflanzenarten auf und lassen sich dadurch nur in Gruppen zusammenfassen (Ohe und Ohe 2007) (s. Abbildung 2b Steinobst).

1.5.2 Pestizidanalytik

Durch den Einsatz von Pflanzenschutzmitteln in der Umwelt, sind Pestizide in vielen Nahrungsmitteln zu finden (Hamilton et al. 2004; Balinova et al. 2006; Meira et al. 2017; Codling et al. 2018; Craddock et al. 2019; EFSA et al. 2021; Liang et al. 2021). Aufgrund der verschiedenen Matrices von Lebensmitteln, wurde von Anastassiades *et al.* (2003) das einfache und schnelle

Aufarbeitungsverfahren QuEChERS (Qu = quick, E = easy, Ch = cheap, E = efficient, R = rugged, S = safe) entwickelt, welches die Isolierung von Pestiziden aus unterschiedlichen Probenmatrices ermöglicht. QuEChERS wird mittlerweile weltweit standardisiert zur Aufarbeitung von Pestiziden verwendet und kann ja nach Probenmatrix individuell angepasst werden (Lehotay et al. 2010; Wilkowska und Biziuk 2011; Koesukwiwat et al. 2014; David et al. 2015; Álvarez-Ruiz et al. 2021; Mekonnen et al. 2021). Die Grundlage bildet eine flüssig-flüssig Extraktion, bei der die Pestizide aus der wässrigen Phase in eine Acetonitril-Phase überführt werden. Anschließend werden die beiden Phasen durch die Zugabe von Salzen getrennt und die Acetonitril-Phase weiter aufbereitet (Anastassiades et al. 2003). Die Probenlösung mit den extrahierten Wirkstoffen aus der Pollenmatrix kann folglich je nach Eigenschaft der Wirkstoffe mittels einer Multimethode mit GC-MS/MS (Gaschromatographie mit gekoppelter Massenspektrometie) und bzw. oder HPLC-MS/MS (High Performance Liquid Chromatographie / Hochleistungsflüssig-chromatographie mit gekoppelter Massenspektrometie) vermessen werden (Kamel 2010; Böhme et al. 2018; Moreno-González et al. 2018; Álvarez-Ruiz et al. 2021).

1.5.3 Mikrobielle Untersuchung

Mikroorganismen in Blütenpollen wurden bislang meist mit kultivierungsbasierten Methoden durch Auszählen von Bakterien- und Pilzkolonien auf Agarplatten bestimmt (Bonvehí und Jordà 1997; González et al. 2005; Estevinho et al. 2012; Feás et al. 2012; Bárbara et al. 2015; De-Melo et al. 2015; Dinkov 2018). In einigen Studien wurden zusätzlich kultivierungsunabhängige Methoden wie die 16S-rRNA Sequenzierung durchgeführt, um die bakterielle Zusammensetzung in frischen und gefrorenen Pollen zu bestimmen (Anderson et al. 2014a; Corby-Harris et al. 2014; Mauriello et al. 2017). Dabei wird nach Extraktion der Bakterien-DNA (Desoxyribonukleinsäure) aus den Pollenproben eine bestimmte Region (V3-V4) der 16S-rRNA Bereiche des Bakterienisolates mit PCR (Polymerase-Kettenreaktion) amplifiziert und anschließend sequenziert und

ausgewertet. Eine neue Studie beschreibt die Isolierung und Bestimmung von Bakterien und Pilzen bzw. Hefen in Blütenpollen (Disayathanoowat et al. 2020). Nach Extraktion der Mikroorganismen-DNA aus den Pollenproben, wurden sowohl Amplikons der V3-V4 Region des bakteriellen 16S-rRNA Gens als auch Amplikons der ITS1 Region im 18S-rRNA fugalen Gen des mit geeigneten Primern sequenziert. Mit Hilfe dieser Methode kann die genaue Zusammensetzung der Bakterien und Pilze bzw. Hefen in Pollenproben bestimmt werden (Disayathanoowat et al. 2020) .

1.5.4 Pyrrolizidinalkaloid-Analytik

PA wurden bislang in unterschiedlichen Lebensmittelmatrices wie Kräuter und Tees, als auch in Honig und Blütenpollen untersucht (Roeder 1995; Boppré et al. 2005; Kempf et al. 2010b; Bodi et al. 2014; Kast et al. 2018; Kaltner et al. 2020). Die PA werden mittels Festphasenextraktion aus der Probenmatrix herausgelöst und anschließend analysiert. Derzeit gibt es zwei verschiedene Möglichkeiten PA zu analysieren: der Nachweis von einzelnen PA mittels LC-MS (Kast et al. 2018) oder die Überführung der 1,2-ungesättigten in einen gemeinsamen Summenparameter mit anschließender GC-MS Detektion (Kempf et al. 2008). Ein empfindlicheres Detektionslimit (bis zu 1 ppb) kann durch die Analyse mit LC-MS/MS erreicht werden, bei der sowohl freie PA als auch die entsprechenden PANO einzeln analysiert werden können (Kast et al. 2018). Starke Probleme der Einzel-Analyse stellt jedoch die geringe Verfügbarkeit von Standardreferenzen dar. Es gibt bislang nur wenige PA oder PANO die als Referenz vorliegen und somit auch genau bestimmt werden können (Dübecke et al. 2011; Gottschalk et al. 2018; Kast et al. 2018). PA bei denen keine entsprechende Referenz vorliegt, können mit einer sogenannten „Ersatz-PA“ quantifiziert werden oder werden während der Analyse nicht mitbestimmt (Betteridge et al. 2005).

2 Ziele der Arbeit

Blütenpollen aus EU- und Nicht-EU Ländern wurden bisher in zahlreichen Studien hinsichtlich möglicher Rückstandsgehalte von Pflanzenschutzmitteln untersucht (DeBiMo 2017-2020; Drummond et al. 2018; Favaro et al. 2019). Die Studien analysierten vorwiegend Mischproben, die wöchentlich oder über eine ganze Saison gesammelt wurden. Einzelne Studien analysierten auch Tagesproben, die stichprobenartig in einer Sammelperiode wöchentlich bzw. monatlich ausgewählt wurden (Böhme et al. 2018). Es ist jedoch wenig über den Eintrag von Pestizidrückständen in täglich gesammelten Proben bekannt. Mit der Hypothese, dass in Pollenproben aus intensiv bewirtschafteten Regionen Pestizidrückstände über eine gesamte Sammelsaison von April bis Juli detektiert werden, war das Ziel der vorliegenden Arbeit, die Verteilung und Entwicklung von Pestizidkontaminationen in täglich gesammelten Blütenpollen während einer gesamten Vegetationsperiode zu untersuchen. Als Grundlage dafür wurden mit Hilfe von Pollenfallen während einer Sammelsaison von April bis Ende Juli 2018 täglich Pollenproben in einer intensiv bewirtschafteten Region in Baden-Württemberg gesammelt. Die Proben wurden auf Wirkstoffe, die derzeit in Deutschland als Pestizide verwendet werden dürfen, analysiert. Damit sollen erste Erkenntnisse über die Anzahl der verschiedenen Pestizide, ihre Häufigkeit und maximale Konzentrationen in Pollen am Beispiel eines Standortes in einer intensiv bewirtschafteten Region erlangt werden.

Weitere Umweltfaktoren wie die mikrobielle Zusammensetzung von Blütenpollen können die Qualität des Produkts maßgeblich beeinflussen (Dinkov 2016, 2018). Bislang wurde die mikrobielle Zusammensetzung sowohl von frisch gesammelten Pollenproben als auch von gelagerten Pollen im Bienenstock in Form von Bienenbrot untersucht (Disayathanoowat et al. 2020). Dabei fehlen vor allem Informationen über die Veränderung von Bakterien- und Pilzgemeinschaften während definierter Lagerbedingungen außerhalb des Bienenstocks. Mit der Hypothese, dass Lagerbedingungen die Zusammensetzung der Mikroorganismen beeinflussen und zum Verderb

führen können, war ein weiteres Ziel dieser Arbeit, qualitative Veränderungen mikrobieller Pollengemeinschaften außerhalb des Bienenstocks zu bewerten. Dazu wurde frisch gesammelter Blütenpollen unter definierten Bedingungen gelagert, um einen möglichen Verderb des Blütenpollens durch Wachstum von lebensmittelverderbenden Mikroorganismen zu simulieren. Die erlangten Ergebnisse sollen dazu beitragen, die entscheidende Bedeutung der Verarbeitung von Blütenpollen durch Kühlung oder Gefrieren nach der Ernte, darzulegen.

Doch nicht nur äußere Einflüsse können die Qualität von Blütenpollen beeinflussen, sondern auch Inhaltsstoffe, die von Pflanzen selbst produziert werden, stellen gewisse Risiken dar (BfR 2013). Bislang ist bekannt, dass ca. 3% aller blühenden Pflanzen weltweit sekundäre Pflanzenmetabolite wie PA produzieren, um sich vor Fressfeinden zu schützen (Smith und Culvenor 1981). Einige Untersuchungen zeigen bereits, dass sowohl in Lebensmitteln mit direktem pflanzlichem Hintergrund wie Kräuter und Tees, aber auch in Bienenprodukten wie Honig und Blütenpollen PA zu finden sind (Roeder 1995; Bodi et al. 2014; Gottschalk et al. 2018). Seither wurden einige kommerzielle Pollenproben aus EU- und Nicht-EU Ländern untersucht (Kempf et al. 2010a). Kontaminationen von PA in saisonal gesammelten Pollenproben zeigte bislang nur eine Studie aus der Schweiz (Kast et al. 2018). Detaillierte Informationen über PAs in Pollenproben von heimischen Pflanzen in Deutschland sind nur vereinzelt zu finden. Mit der Hypothese, dass PA sich auf PA-produzierende Pflanzen zurückführen lassen, war weiteres Ziel dieser Arbeit ein ausführliches Wissen über die PA Kontaminationen in saisonalem Blütenpollen aus Süddeutschland zu erlangen. Dazu wurden Mischproben von Blütenpollen im Juli 2019 an 57 Standorten in Baden-Württemberg gesammelt. Die Proben wurden mittels palynologischer Analyse auf deren Pollenzusammensetzung untersucht, sowie die Gehalte an PA analysiert. Mit Hilfe der Ergebnisse sollen Pflanzen identifiziert werden, die für hohe PA Gehalte in den Proben verantwortlich sind und eine Risikobewertung der gefundenen PA Gehalte durchgeführt werden.

Die Untersuchungen sollen dabei helfen, weitere Erkenntnisse sowohl über die Belastung durch PSM als auch über die Zusammensetzung heimischer Blütenpollen und die Verwendbarkeit hinsichtlich der Nutzung als Lebensmittel zu erlangen.

3 Veröffentlichungen

3.1 Pesticide residues in daily bee pollen samples (April-July) from an intensive agricultural region in Southern Germany

Publiziert in *Environmental Science and Pollution Research:*

Abstract

Insect-pollinated plants are essential for honey bees to feed their brood. In agricultural landscapes, honey bees and other pollinators are often exposed to pesticides used for cultivation. In order to gain more insight to the fluctuation of pesticide loads, 102 daily pollen samples were collected between April and July 2018 in a fruit-growing area in Southern Germany. Samples were analyzed with respect to more than 260 pesticides using a multi-residue pesticide analysis method. Almost 90% of the analyzed pollen samples featured between one and thirteen different pesticides. In total, 29 pesticides were detected at maximum concentrations of up to 4500 ng/g pollen. Maximum residual concentrations of most pesticides were observed during April and the first half of May, as well as during second half of June. In most cases, serial data of pesticide residuals were detected for approximately 10 subsequent days with two or three maximum values, which were several folds higher than concentrations on the days before and thereafter. The pollen hazard quotient (PHQ) was calculated to estimate the risk of the detected pesticides to honey bees and wild pollinators.

Friedle, C., Wallner, K., Rosenkranz, P., Martens, D., Vetter, W. (2021): Pesticide residues in daily bee pollen samples (April-July) from an intensive agricultural region in Southern Germany. In: *Environ Sci Pollut Res,* S. 1-15. DOI: 10.1007/s11356-020-12318-2.

Environmental Science and Pollution Research
https://doi.org/10.1007/s11356-020-12318-2

RESEARCH ARTICLE

Pesticide residues in daily bee pollen samples (April–July) from an intensive agricultural region in Southern Germany

Carolin Friedle[1] · Klaus Wallner[1] · Peter Rosenkranz[1] · Dieter Martens[2] · Walter Vetter[3]

Received: 7 July 2020 / Accepted: 30 December 2020

Abstract
Insect-pollinated plants are essential for honey bees to feed their brood. In agricultural landscapes, honey bees and other pollinators are often exposed to pesticides used for cultivation. In order to gain more insight into the fluctuation of pesticide loads, 102 daily pollen samples were collected between April and July 2018 in a fruit-growing area in Southern Germany. Samples were analyzed with respect to more than 260 pesticides using a multi-residue pesticide analysis method. Almost 90% of the analyzed pollen samples featured between one and thirteen different pesticides. In total, 29 pesticides were detected at maximum concentrations of up to 4500 ng/g pollen. Maximum residual concentrations of most pesticides were observed during April and the first half of May, as well as during the second half of June. In most cases, serial data of pesticide residuals were detected for approximately 10 subsequent days with two or three maximum values, which were several folds higher than concentrations on the days before and thereafter. The pollen hazard quotient (PHQ) was calculated to estimate the risk of the detected pesticides to honey bees and wild pollinators.

Keywords Bee pollen · Pesticide residues · Pollen hazard quotient · QuEChERS · LC-MS/MS · Germany

Introduction

Apart from nectar, pollen from plants is essential to honey bees (*Apis mellifera)* for feeding their brood. From early spring on, bees start collecting pollen from blooming crops, e.g., willows, fruits, vegetables, and flowers, and transporting it in the pollen baskets known as corbicula via their legs to the hive (Kevan and Baker 1983; Willmer 2011). After the addition of nectar and bee secretion, pollen is stored in comb cells (Nagai et al. 2005; DeGrandi-Hoffman et al. 2013). The stored pollen, called bee bread, can be stored over months, but bees prefer to consume freshly stored pollen within 2 to 4 days (Anderson et al. 2014; Carroll et al. 2017). Bee bread is then used by nurse bees to produce larval food (Lindauer 1952; Cridge et al. 2015). However, bee bread and bee pollen were shown to be frequently contaminated by pesticides (Lindauer 1952; Chauzat et al. 2006; Botías et al. 2015; Traynor et al. 2016; Codling et al. 2018; Böhme et al. 2018, 2019; German Bee Monitoring 2014–2019). This is due to the fact that honey bees are collecting nectar and pollen not only from wild plants but mainly from crops or plants used in agricultural industry (McGregor 1976).

Residues of agricultural pesticides in pollen can originate from the application of systemic compounds before the blooming period, from contamination of water and soil as well as from spray application to the blooming plants (Aktar et al. 2009; The Scottish Government 2018). To protect orchards and oilseed against pests and fungal growth, spray applications of "pesticide cocktails" are recommended in Germany with spraying regimes of up to 15 different applications from early spring to late summer (Wallner 2012; Roßberg and Harzer 2015). The contamination status of bee pollen can be monitored by means of a pollen trap installed at the front of a hive entrance which samples up to 40% of the daily amount of pollen brought to the hive by foraging bees (Keller et al. 2005). Most studies to date have been performed on pooled samples, typically collected from weekly trappings, at single

Responsible Editor: Philippe Garrigues

✉ Carolin Friedle
carolin_friedle@uni-hohenheim.de

[1] Apicultural State Institute, University of Hohenheim, Stuttgart, Germany

[2] Agricultural Research and Development Institute, Speyer, Germany

[3] Institute of Food Chemistry (170b), University of Hohenheim, Stuttgart, Germany

Published online: 11 January 2021

timepoint from multiple colonies in the same apiary, or multiple collections are pooled over an entire season (Drummond et al. 2018; Tosi et al. 2018). Little is known about the pesticide load in daily samples of bee pollen. Böhme et al. (2018) analyzed in between 9 and 39 of daily bee pollen samples collected from March to August over a time course of 5 years, at three different agricultural sites ("meadow" with about 60% permanent grassland, "grain" with high percentages of grains, and "fruit" with 30% permanent crops) in Southern Germany. Random analysis of at least one sample each week indicated highest pesticide concentrations at the "fruit" site with 7200 ng/g pollen. These concentrations were much lower than those reported in two studies from the USA where pesticide concentrations in weekly pollen samples mounted up to 99,000 ng/g pollen (Mullin et al. 2010; Stoner and Eitzer 2013). Likewise, the pesticide load in bee pollen could be traced back to pesticide spraying in different cultivation areas. Nevertheless, detailed knowledge about the development of pesticide concentrations in pollen samples during a spring collecting season is still lacking. Furthermore, frequency and time course of remaining pollen contamination was vastly unknown for the time period after occurrence of the maximum contamination level.

The goal of this project was to study the distribution and progression of pesticide contamination in daily bee pollen samples throughout an entire growing season (April to July 2018) at a representative bee hive located within a fruit cultivation area in Southern Germany. For this purpose, daily samples were collected from a single hive by means of a pollen trap from April to July 2018. Samples were analyzed for over 260 pesticides by LC-MS/MS, including almost all as generally used in Germany. The data collected was used to elucidate number of different pesticides, their frequency, and maximum concentrations in pollen, as well as record their reoccurrence during the entire growing season to understand pesticide fluctuations in an agricultural landscape.

Materials and methods

Site description and collection One individual bee colony was used for sampling. Since a colony usually consists of more than 30,000 bees, typically ~ 20% of the workers are engaged in foraging pollen on a given day (Klein et al. 2019). This scenario seemed to be appropriate in order to get first impressions about the amount of pesticides honey bees become daily exposed to over an entire season. An apiary on personal grounds was selected for this study (exact coordinates of the apiary will not be shown and no permits were needed for this study); it is located in an intensive fruit cultivation area nearby Friedrichshafen (Baden-Wuerttemberg, Southern Germany) (Fig. 1). The area around the hive within the mean foraging distance of foraging bees of around 1500 m (Steffan-Dewenter and Kuhn 2003) is characterized by a low population density (population 325/km^2) (Statistical Service Office Baden-Wuerttemberg 2020) and cultivation of different crops. Apples, sweet and sour cherries, and plums with over 0.25 km^2 agricultural area (Info Service of Agricultural - Nutrition and Rural Areas 2018; Statistical Service Office Baden-Wuerttemberg 2017) are cultivated in this area. Bee pollen traps were installed to collect pollen loads from returning honey bees (*Apis mellifera*) (Detroy and Harp 1976) (Figure S1). From April (starts at April 12 with numeric D1) until July 31 (D102), 2018, daily pollen samples were collected on 102 out of 111 consecutive days (except nine rainy days without foraging activities) by a volunteer beekeeper. Daily bee pollen samples (20 to 55 g) were homogenized, and aliquots of 20 g were removed and stored at − 20 °C in polyethene sample bags until sample preparation.

Chemicals Acetonitrile (for LC-MS, ≥ 99.95%), nonane (for synthesis, ≥ 99%), and fine magnesium sulfate (≥ 99%) were from Carl Roth (Karlsruhe, Germany). The sorbent Sepra C_{18}-E (50 µm, 65 Å) and Sepra PSA (51 µm, 73 Å); the "roQ QuEChERS Kit" with 4.0 g magnesium sulfate, 1.0 g sodium chloride, 1.0 g sodium citrate tribasic dihydrate, and 0.5 g sodium citrate dibasic sesquihydrate; and Sepra graphitized carbon black (laboratory use) were from Phenomenex (Karlsruhe, Germany). The internal standard solution was prepared in-house at the Agricultural Research Development Institute (LUFA Speyer Germany) with triphenyl phosphate and d_{10}-chlorpyrifos (20 ng/µL acetonitrile each) (HPC, Bohnsdorf, Germany). Also, the pesticide standard solution with all 262 analytes (HPC, Bohnsdorf, Germany, and Sigma-Aldrich, Darmstadt, Germany) at concentration 1 ng/µL acetonitrile (Table S1) was prepared at the Agricultural Research Development Institute (LUFA, Speyer).

Sample preparation for pesticides analysis Pollen samples were brought up to room temperature, mechanically homogenized in a mortar, and subsequently dried for 8 h at 30 °C in a heating cabinet (Binder, Tuttlingen, Germany). An aliquot of 5 g ± 0.001 g pollen sample was weighed into a 50-mL tube (Buddeberg, Mannheim, Germany), and the following QuEChERS method (Anastassiades et al. 2003) (§64 LFGB, BVL L 00.00-115/1:2015-03) with individual modifications was used. Ten milliliter demineralized water, 10 mL acetonitrile, and 10 µL internal standard solution were added, and the mixture was mechanically shaken for 20 min at 400 rpm on a Heidolph Instruments Promax 2020 (Schwabach, Germany). After centrifugation (10 min at 2750 x G), the supernatant was transferred into a new 50-mL tube, which contained 4.0 g magnesium sulfate, 1.0 g sodium chloride, 1.0 g sodium citrate tribasic dihydrate, and 0.5 g sodium citrate dibasic

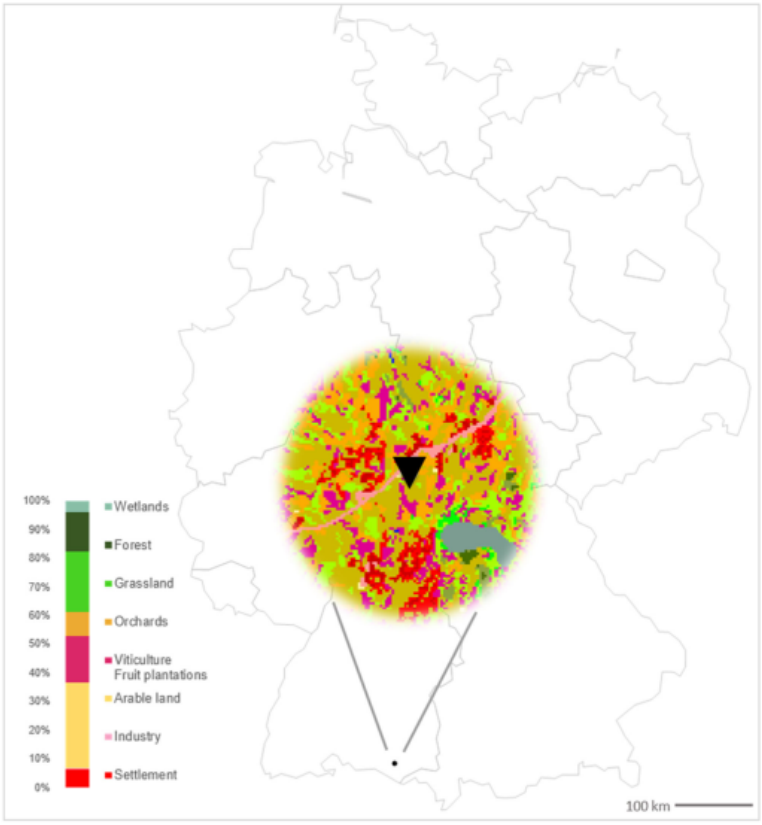

Fig. 1 Location area of sampling (r = foraging distance 1500 m) in Southern Germany (mapping with JMP® pro 15.0; landscape use: basis data from the Environmental Information System (UIS) of the State Institute for the Environment Baden-Wuerttemberg; evaluation with GIS, geonline GmbH)

sesquihydrate (roQ QuEChERS Kit). The sample solution was shaken for 2 min followed by centrifugation for 10 min at 2750 x G. The supernatant was placed in a 15-mL tube (Buddeberg, Mannheim, Germany) which already contained 0.5 g fine $MgSO_4$. The sample was shaken for 1 min and centrifuged (10 min at 2750 x G). Then, 5 mL of the supernatant was transferred into a glass tube and supplemented with 50 µL nonane solution (10 g nonane/50 mL acetone). Afterwards, the sample solution was evaporated to near dryness in a heating block maintained at 40 °C under nitrogen flow. The residue was resuspended in 2.5 mL acetonitrile and swiveled for 30 s in an ultrasonic bath. The solution was then transferred into a new 15-mL tube containing 0.49 g cleaning mixture (0.31 g magnesium sulfate, 0.06 g Sepra C_{18}-E, 0.11 g Sepra PSA, and 0.01 g graphitized carbon black). The sample was shaken for 1 min and centrifuged for 10 min at 2750 x G. A 1 mL aliquot from the supernatant was transferred into a 1.5 mL LC vial (Macherey-Nagel, Düren, Germany) and stored at − 20 °C until analysis.

Palynological analysis A palynological analysis of four selected samples D9-D13 (April 22–26) was prepared as follows. An aliquot of 100 mg homogenized pollen was weighed into a 50-mL tube containing 10 mL demineralized water and a drop of dish soap. The mixture was shaken for 1 min, and a drop was transferred to an object carrier, dried, and covered with Kaiser's glycerol gelatin for microscopy (Merck, Darmstadt, Germany).

High performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis of pesticides LC-MS/MS analyses were performed with an API 400 system (AB Sciex, Darmstadt, Germany) at Agricultural Research and Development Institute (LUFA, Speyer, Germany). Analytes were separated on a Gemini NX C_{18} column (100 mm length × 3 mm inner diameter, 3 µm particle size; Phenomenex, Karlsruhe, Germany) at 30 °C. Each 10 µL of sample was injected to the column equilibrated with 5 mmol/L ammonium acetate and 0.1% formic acid in water (A) at a flow rate of

300 µL/min. A linear gradient, within 3 min, of 30 to 70% (B) (methanol, 5 mmol/L ammonium acetate) in A was run, followed by an increase to 100% (B) within 10 min. After 2 min at 100% (B), the ratio was turned back to 70% and held for another 5 min. MS/MS measurements were performed in multiple reaction monitoring (MRM) mode using electrospray ionization (ESI) in the positive mode with an ion spray voltage of 5500 V and a desolvation temperature of 400 °C. A total of 262 analytes (pesticides and related compounds) were subjected to this analytical method (Table S1).

Quality control One sample fortified with analytes was run per batch of 25 samples in a following manner: blank pollen (5 g pollen sample without detectable residues, as checked previously) was mixed with 10 mL demineralized water and 9.75 mL acetonitrile, spiked with 250 µL pesticide standard solution and 10 µL internal standard solution (absolute concentration 200 ng triphenyl phosphate and d_{10}-chlorpyrifos each), and prepared as explained above in "Sample preparation for pesticides analysis". All samples were prepared within 1 week, one batch (25 samples + 1 blank pollen) per day. The recovery was calculated using a defined final volume of 2.5 mL (after evaporation) by comparison with the absolute internal standard. Recovery rates were calculated for four quality control samples in total, revealing a range between 29 and 160% per substance (except spirodiclofen, exhibiting a significant low recovery rate of 4%). The mean standard deviation between replicates was 14% (Table S2).

Toxicological evaluation In order to assess the hazard to honey bees of pesticide residues in the pollen samples, the pollen hazard quotient (PHQ) was used following the method of Stoner and Eitzer (2013). The PHQmax is calculated by dividing the maximum concentration (ng/g) of each pesticide detected in the samples by the known LD_{50} value (honey bee oral; µg/bee) as listed in the University of Hertfordshire pesticides properties database (Pesticide Properties DataBase - PPDB 2020). The total PHQ per day (tPHQday) was calculated as the sum of all PHQs of pesticides in the representative day sample. Based on a daily consumption of up to 9.5 mg bee bread by a nurse bee (Rortais et al. 2005), a PHQ of ≥ 50 was considered "relevant" for bee health according to Böhme et al. (2018). With a PHQ of 100, 0.1% of the LD_{50} would be ingested in 1 day, or 1% of the LD_{50} in a 10-day nursing period (Stoner and Eitzer 2013).

Results

General observations

Between April and July 2018, 102 daily pollen samples were collected and analyzed for 262 active substances (75 fungicides, 95 herbicides, 89 insecticides, and 3 plant regulators). Altogether 29 pesticides were detected, 15 fungicides, 12 insecticides, and 2 herbicides (Table 1), while the other 258 compounds for which we screened were not detected in any sample. Only 13 daily pollen samples contained no detectable pesticide residues, whereas 89 pollen samples contained between one and thirteen residues per sample (Table S3). The median number of detected residues was five per daily sample. More detected residues, between seven and thirteen per sample, were found from D8 to D17 (April 21–30). Of the 29 individual pesticides detected, these were found in daily pollen samples anywhere on 3 to 64 occasions. The median frequency of detection was 14. The greatest frequency of detection of 64 (d_f in days) was observed for the fungicide trifloxystrobin (Figure S2). The maximum concentrations of the 29 positive detected pesticides ranged between 6 and 4530 ng/g pollen, while tebuconazole showed the greatest concentration of all pesticides with 4530 ng/g pollen in one sample (Table 1). Furthermore, the highest total pesticide concentrations per day sample, between 3300 and 8800 ng/g pollen, occurred in samples between D10 and D12 (Table S3). The day-to-day progression of individual pesticide concentrations in pollen samples is discussed in the following subchapters. Pesticides were divided into their classes of fungicides, herbicides, and insecticides.

Fungicides

A total of 15 fungicides were detected in all pollen samples. The two highest concentrations measured in this study showed tebuconazole and fluopyram with concentrations above 4000 ng/g pollen.

Tebuconazole (d_f 17). The greatest single day concentration of 4530 ng/g pollen was observed on D10 (April 23, 2018) (Fig. 2a). This triazole fungicide, normally used against various foliar diseases such as powdery mildew and black spot in fruit and vegetable cultivation and in crop seed (Federal Office of Consumer Protection and Food Safety 2020) (Table 1), was detected in 17 daily pollen samples. The highest values were observed on 10 subsequent days between D7 and D16. Initially detected on moderate concentrations between D7 and D8, tebuconazole concentrations slightly increased on D9 to 230 ng/g pollen. On the following day, a maximum concentration of 4530 ng/g was measured. After 2 consecutive days at a high level, tebuconazole concentrations fell sharply until it was no longer detectable on D17. At the end of May (D41), tebuconazole was detected on a second occasion at 160 ng/g pollen in just one daily pollen sample.

Fluopyram (d_f 27) is a succinate dehydrogenase inhibitor used in fruit and vegetable cultivation and viticulture (Table 1) and was detected in 27 daily pollen samples. It is noted that the greatest concentration of 4050 ng/g pollen was recorded on the same day as the maximum observed with tebuconazole

Table 1 Summary of 29 pesticides detected in 102 daily bee pollen samples during April–July 2018 in Southern Germany

Substances	Class[a]	Group	Application area in Germany[b]	LOQ (ng/g)	Recovery rate (%)	Frequency of detection (d_f)	Max. detected concentration (ng/g)	Mean detected concentration (ng/g)	LD_{50} (μg/bee)[c]	PHQmax	Mean PHQ
Acetamiprid	I	Neonicotinoids	f; v	3	84	11	31	1.3	15	2.1	0.09
Azoxystrobin	F	Quinone outside inhibitors	f; v	3	123	6	14	0.6	25	0.6	0.03
Boscalid	F	Succinate dehydrogenase inhibitors	f; v; r	3	105	36	201	7.9	166	1.2	0.05
Chlorantraniliprole	I	Diamides	f; v	3	120	25	307	13.6	104	3.0	0.13
Cyprodinil	F	Anilino-pyrimidines	f	3	29	9	193	5.8	113	1.7	0.05
Difenoconazole	F	Demethylation inhibitors	f; v	3	87	10	48	1.5	177	0.27	0.01
Diflubenzuron*	I	Chitin synthesis inhibitors	f; v; c (B1)	3	79	2	121	1.3	9.1	13	0.14
Dimethenamid	H	Chloroacetamides	f; v; c	3	98	11	121	2.5	118	1.0	0.02
Dimoxystrobin	F	Quinone outside inhibitors	r	3	108	2	6.1	0.1	79	0.08	0.001
Fenhexamid	F	Sterol biosynthesis inhibitors	f; v	10	44	10	274	6.3	102	2.7	0.06
Fenoxycarb*	I	Insect growth regulator	f; c (B1)	3	96	14	367	5.0	204	1.8	0.02
Fenpyroximate	I	Pyrazole	f; v; w	3	82	15	99	2.6	119	0.8	0.02
Flonicamid	I	Flonicamid	f; v; r	3	83	19	35	2.4	100	0.4	0.02
Fluopyram	F	Succinate dehydrogenase inhibitors	f; v; w	5	125	27	4050	123	102	40	1.2
Kresoxim-methyl	F	Quinone outside inhibitors	w	5	116	3	10	0.3	110	0.09	0.002
Methiocarb	I	Carbamates	s.t.	3	94	16	14	1.0	0.08	170	12.2
Myclobutanil	F	Demethylation inhibitors	f; v; w	5	106	39	334	11.9	34	9.8	0.4
Penconazole	F	Demethylation inhibitors	f; v; w	3	55	14	24	1.2	112	0.2	0.01
Pendimethalin	H	Dinitroanilines	c	5	53	17	1810	36.6	101	18	0.4
Picaridin	I	Piperidines	i.r.	15	82	15	117	5.8	–	–	–
Pirimicarb	I	Carbamates	f; v; c	3	53	3	25	0.5	4.0	6.3	0.14
Pyraclostrobin	F	Quinone outside inhibitors	f; v; c	5	100	6	49	1.1	110	0.44	0.01
Pyrimethanil	F	Anilino-pyrimidines	f; w; c	5	57	9	52	1.8	100	0.52	0.02
Spirodiclofen	I	Biosynthesis inhibitors	f; v; c (B1)	5	4	29	402	15.8	196	2.1	0.08
Tebuconazole	F	Demethylation inhibitors	f; v; w	5	90	17	4530	98.4	83	55	1.8
Tebufenozide	I	Diacylhydrazines	f; v; w	3	107	18	412	8.7	100	4.1	0.09
Thiacloprid	I	Neonicotinoids	f; v; c	3	77	47	258	14.0	17	15	0.8
Thiophanate-methyl	F	b-Tubulin inhibitors	f; c	10	101	7	59	2.1	115	0.51	0.02
Trifloxystrobin	F	Quinone outside inhibitors	f; v; c	3	112	64	707	40.6	110	6.4	0.37

*, not approved for application in Germany; –, not available

[a] *F* fungicide, *H* herbicide, *I* insecticide

[b] *c* crop seed, *f* fruit cultivation, *i.r.* insect repellent, *r* rapeseed, *s.t.* seed treatment, *v* vegetable cultivation, *w* wine, *B1* is classified as hazardous to bees (Federal Office of Consumer Protection and Food Safety 2020)

[c] LD_{50} honey bees oral (*Apis* sp.), values rounded to two significant numbers (Pesticide Properties DataBase - PPDB 2010)

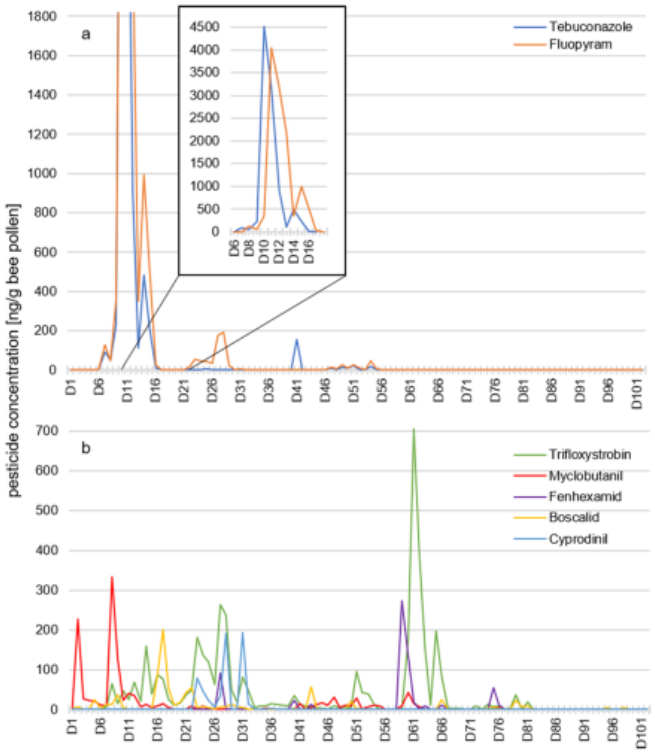

Fig. 2 Line chart with a day-to-day progression of fungicides with maximum concentrations (**a**) > 4000 ng/g bee pollen and (**b**) between 100 and 700 ng/g bee pollen

(D10). This observation and the very similar progress over the same period (Fig. 2a, Figure S3) suggested that both fungicides were possibly applied together. Specifically of note in this context, there is at least one formulation used in Germany for fruit cultivation (Federal Office of Consumer Protection and Food Safety 2020) containing fluopyram and tebuconazole, both with concentrations of 200 g/L. A palynological sample analysis of pollen for days with highest load of fluopyram and tebuconazole (D10 and D11) confirmed high proportions of *Prunus* sp. type (± 77% stone fruit) and *Pyrus* sp. type (± 18% pome fruit) along with scattered contributions of *Acer* sp. (maple) and *Picea* sp. (spruce) pollen (Table S4). Hence, there exists strong evidence that both pesticides were also distributed and collected at similar ratio during fruit cultivation. In the beginning of May, fluopyram was detected again on 10 subsequent days (D22 to D31). However, the maximum concentration of 190 ng/g pollen was much lower (Fig. 2a), suggesting either the spraying of a lower amount of fluopyram or a lower share of pollen from the treated field in the daily collection of the hive. The absence of tebuconazole in these samples pointed towards the use of a different pesticide formulation. Within this period, the highest concentration was reached on the seventh day.

Trifloxystrobin (d_f 64) is mainly used in fruit and vegetable cultivation and crop seed (Table 1) and was found most frequently of all pesticides, showing a frequency of detection of 64 out of 102 days with a maximum concentration of 710 ng/g pollen. It was detected consistently between D7 and D49 with only one exception. Within this period, the concentrations increased and dropped again (concentration up to 260 ng/g pollen). Between D22 and D29, trifloxystrobin showed similar concentrations as fluopyram (in its second period). While the resulting constant ration of both fungicides could be accidental, it could also mean that they were applied together. Formulations containing fluopyram together with trifloxystrobin are used in fruit cultivation in Germany (both with concentrations of 250 g/L (Federal Office of Consumer Protection and Food Safety 2020)). The long period and the differing composition provide evidence for application of this fungicide on several days at different locations. In addition, a second period with higher trifloxystrobin levels was observed between D60 and D70, with maximum concentration of 710 ng/g pollen in one sample. It required 9 subsequent days for the concentration to fall below LOQ (Fig. 2b).

Myclobutanil (d_f 39) is frequently applied as a demethylation inhibitor in fruit and vegetable cultivation and viticulture.

It was first observed in this investigation in the samples between D2 and D18. Within these 17 days, the concentrations were usually low, except on D2 (230 ng/g pollen) and D8 (maximum level of 330 ng/g pollen) (Fig. 2b). Again, subsequent detections suggested repeated applications either on the same field or on other local areas. *Fenhexamid (d_f 10)*, which also had a low recovery of 44%, was detected between May and July at moderate and lower concentrations. The highest concentrations were measured between D59 and D66, including 4 days showing up to 270 ng/g pollen. Detection of *boscalid (d_f 36)* started at a low level in April with up to 200 ng/g pollen on D17, followed by a concentration drop. *Cyprodinil (d_f 9)* displayed a low recovery rate of only 29%. Nevertheless, this aminopyrimidine fungicide peaked at 190 ng/g pollen on D28 and was detected at moderate concentrations between D23 and D27 (Fig. 2b).

Further eight fungicides with concentrations < 100 ng/g pollen could be detected in the samples. The majority of these substances (*thiophanate-methyl (d_f 7)*, *difenoconazole (d_f 10)*, *pyraclostrobin (d_f 6)*, *pyrimethanil (d_f 9)*, *azoxystrobin (d_f 6)*, *kresoxim-methyl (d_f 3)*, and *dimoxystrobin (d_f 2)*) showed highest concentrations between D2 and D28. *Penconazole (d_f 14)* was detected between D58 and D62 and again between D75 and D78 at lower concentrations (Figure S4a).

Herbicides

Only the following two herbicides were detected with maximum concentrations between 120 and 1810 ng/g pollen in the analyzed pollen samples.

Pendimethalin (d_f 17) is commonly used in vegetable cultivation and crop seed conditioning. It was detected in 17 samples. The analytical recovery of pendimethalin was comparably low (55%, standard deviation 9.46%) (Table 1, Table S2). Since these results could not be reliably confirmed, the actual pendimethalin concentrations may have been underestimated by almost a factor of two. After four subsequent positive findings in early June (D43 to D46) with up to 150 ng/g pollen, pendimethalin was detected again with high abundance on D65 and D66 at 1810 and 950 ng/g pollen. After a gap of 4 days, pendimethalin was detected again between D71 and D81 (maximum concentration of 330 ng/g pollen) (Fig. 3a). *Dimethenamid (d_f 11)* was detected in five unconnected daily pollen samples exhibiting lower concentrations in May, followed by two daily samples from June (D65 and D66) showing a maximum concentration of 120 ng/g pollen.

Insecticides

In total, 12 insecticides were detected in all analyzed samples. Seven insecticides were observed with maximum concentrations between 100 and 415 ng/g pollen.

Tebufenozide (d_f 18) was detected in 18 daily pollen samples. The first period lasted from D7 to D18 (maximum concentration of 410 ng/g pollen on D8). Two days later, tebufenozide was detected again but at much lower concentrations between D21 and D27, except one sample (Fig. 3b). Also, in this case, the period of persistence lasted for about 10 days.

The inhibitor of lipid biosynthesis *spirodiclofen (d_f29)* is mainly used as an acaricide and insecticide in fruit cultivation along with vegetable cultivation and crop seed treatment and is classified as hazardous to bees (Table 1). In contrast to a reported recovery rate of 70% in potatoes (Attallah et al. 2012), this study showed a calculated recovery rate of only 4% in pollen samples (61% standard deviation). These measured spirodiclofen concentrations were not considered reliable for a detailed evaluation, and only those periods in which spirodiclofen was detected were reported. Spirodiclofen was detected between D40 and D47 and then again between D49 and D63 and after one negative sample, with highest concentration measured between D65 and D66. Between D74 and D78, spirodiclofen was detected at lower level on 5 days (Fig. 3b).

Fenoxycarb (d_f 14) was detected in 14 inconsecutive day samples between D43 until D81; its concentrations were typically low, except for a maximum concentration of 370 ng/g pollen at D53. Fenoxycarb is an insect growth regulator used to control scale insects on fruits or other plants and is not approved for application to blooming crops in Germany (Pesticide Properties DataBase - PPDB 2020), because it is classified hazardous to bees and can have negative effects on bee brood (Czoppelt 1991; Aupinel et al. 2007). It could contaminate pollen by drifting onto flowers and herbs in the proximity of application on non-flowering crops.

Chlorantraniliprole (d_f 25) was detected between D43 and D61 at moderate to low concentrations (e.g., 110 ng/g pollen at D50) except for 1 day. Similarly, the second period lasted from D74 to D81 and featured one daily pollen sample with a maximum value of 310 ng/g pollen on D75 (Fig. 3b, Table 1). The neonicotinoid *thiacloprid (d_f 47)* was one of the most frequently detected pesticides. Apart from one sample, it was continuously present in daily pollen samples from D1 to D49. Only for 2 days in April (D2 and D8), *thiacloprid* concentrations exceeded 200 ng/g pollen. The chitin synthesis inhibitor *diflubenzuron (d_f 2)* is not approved for application to blooming crops in Germany and was detected in two daily samples from D7 to D8, displaying widely varying concentrations of 120 and 10 ng/g pollen, respectively. Finally, *picaridin (d_f 15)* which is generally used as an insect repellent by humans and not as an agricultural pesticide was detected at low levels between D40 and D46. The maximum concentration of 120 ng/g pollen picaridin was determined during the last days of the study, referring

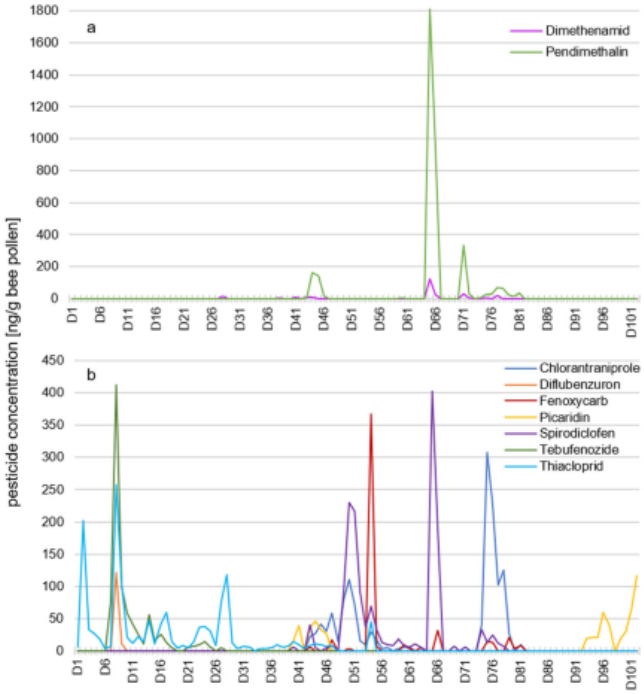

Fig. 3 Line chart with a day-to-day progression of (**a**) herbicides with maximum concentrations and (**b**) insecticides with maximum concentrations between 100 and 400 ng/g bee pollen

to the period from D93 to D102 (Table S3). The beekeeper who collected the daily pollen samples could have been the unintended source of the picaridin contamination in the pollen samples, because he confirmed utilization of a potentially relevant insect repellent during summer.

Further five insecticides were detected with comparable low concentrations of < 100 ng/g pollen. *Fenpyroximate (d_f 15)*, *flonicamid (d_f 19)*, *acetamiprid (d_f 11)*, and *methiocarb (d_f 16)* showed their highest concentrations between D2 and D28, while *pirimicarb (d_f 3)* was detected between D58 and D62 and again between D75 and D78 but only at lower concentrations (Figure S4b, Table S3).

It remained unclear whether the individual components were interrelated. An exemplary hierarchical cluster analysis (JMP® pro 15.0) was performed in order to show a possible connection between the presented insecticides (and further fungicides with concentrations < 100 ng/g pollen). Only one single day-to-day progression of the insecticides fenpyroximate and flonicamid is shown between D1–17, D53–58, and D101. There is no formulation used in Germany in which these two pesticides are applied together. For all other substances, no similar course between the day-to-day progressions could be established (Figure S5).

Pollen hazard quotients at maximum concentrations

This study clearly demonstrates that pesticide levels in daily pollen samples varied strongly. Within a typical period of 10 days, concentrations peaked on 1 or 2 days and then leveled out. It was aimed to estimate the measured highest threat presented by the pesticide at maximum concentration (PHQmax). PHQmax values of the 29 detected pesticides ranged from 0.09 to 170 (Table 1). The peak value of 170 could be allocated to the insecticide methiocarb, although its corresponding maximum concentration was only 14 ng/g pollen. However, LD_{50} of methiocarb was determined corresponding to a very low level of 0.08 µg/bee (Table 1). Tebuconazole also showed a high PHQmax of 55, linked to the highest individual pesticide concentration determined in any of the samples. The tPHQday (calculated as the sum of all PHQs per sample and day) represents the total pesticide load per day. Altogether four samples revealed a tPHQday value between 100 and 180 (D6 to D8; D41), and 15 daily samples exceeded a value over 50 (Table 2). Thiacloprid was the most frequently trafficked pesticide in these samples but had comparably low PHQ values below 10 in all reported samples. In addition, methiocarb, myclobutanil, trifloxystrobin, boscalid, flonicamid, and fluopyram showed resulting PHQ values in

Table 2 Daily pollen samples with tPHQday values > 50, with total pesticide concentrations and their calculation

Sampling day	Total pesticide detects	tPHQ	Total pesticide conc. per sample (ng/g)	Thiacloprid (PHQ/pesticide conc. (ng/g))	Methiocarb (PHQ/pesticide conc. (ng/g))	Myclobutanil (PHQ/pesticide conc. (ng/g))	Trifloxystrobin (PHQ/pesticide conc. (ng/g))	Boscalid (PHQ/pesticide conc. (ng/g))	Flonicamid (PHQ/pesticide conc. (ng/g))	Fluopyram (PHQ/pesticide conc. (ng/g))	Tebuconazole (PHQ/pesticide conc. (ng/g))	Tebufenozide (PHQ/pesticide conc. (ng/g))	Pyrimethanil (PHQ/pesticide conc. (ng/g))
D2	7	90	571	11.6	70.1	6.77		0.05	0.25				
				201	5.61	227		7.5	25.2				
D5	6	51	88	1.1	49.7	0.6		0.15	0.13				
				18.9	3.89	20.4		25.4	13.1				
D6	5	174	53	0.2	174	0.34		0.02	0.19				
				3.99	13.9	11.5		3.22	19.2				
D7	10	142	387	0.4	138	0.35	0.06	0.08	0.35	1.23	1.1	0.75	
				75	11.0	12.0	7.0	14.0	35.0	126	92	75	
D8	13	183	1484	14.9	138	9.9334	0.59	0.08	0.13	0.46	0.59	4.12	0.52
				258	11.0		65.0	13.0	13.4	47.0	49.0	412	52.0
D10	10	98	8837	1.2		0.68	0.44	0.04	0.12	39.6	54.5	0.58	0.46
				21.0		23.0	48.0	6.0	12.0	4046	4527	58.0	46.0
D11	10	80	6517	0.7	7.4	1.2	0.24		0.15	31	38.2	0.41	0.24
				12.0	0.59	41.7	26.0		15.0	3169	3171	41.0	28.0
D14	11	68	1851	2.3	46.1	0.37	1.5		0.18	9.7	5.9	0.55	0.09
				47.1	3.69	12.5	159		17.9	994	485	55.4	8.98
D15	8	50	827	0.7	40.9	0.15	0.35		0.09	5.2	2.6	0.16	
				11.4	3.27	5.0	38.4		8.55	525	217	16.4	
D16	13	67	335	2.4	61.5	0.25	0.8	0.51	0.06	0.24	0.10	0.25	0.07
				41.3	4.92	8.58	88.0	84.2	5.77	24.7	8.3	25.3	6.99
D20	7	53	77	0.5	51		0.16	0.12					0.06
				9.13	4.08		17.6	19.5					5.53
D24	11	68	330	2.2	62.6	0.26	1.2	0.04		0.47		0.15	
				37.5	5.0	8.79	136	6.44		48.3		14.9	
D25	9	71	249	1.8	66.6		1.1	0.03		0.43	0.07	0.07	
				30.7	5.33		119	5.15		44.5	5.73	6.5	
D28	11	53	787	6.8	40.1		2.2	0.04		1.9			0.08
				118	3.2		238	6.71		194			8.36
D41	7	166	268	0.6	162	0.43	0.12				1.9		
				10.3	13	14.5	13.5				156		

Sampling day	Difenoconazole (PHQ/pesticide conc. (ng/g))	Fenpyroximate (PHQ/pesticide conc. (ng/g))	Acetamiprid (PHQ/pesticide conc. (ng/g))	Cyprodinil (PHQ/pesticide conc. (ng/g))	Thiophanate-methyl (PHQ/pesticide conc. (ng/g))	Azoxystrobin (PHQ/pesticide conc. (ng/g))	Dimethenamid (PHQ/pesticide conc. (ng/g))	Dimoxystrobin (PHQ/pesticide conc. (ng/g))	Kresoxim-methyl (PHQ/pesticide conc. (ng/g))	Pyraclostrobin (PHQ/pesticide conc. (ng/g))	Diflubenzuron (PHQ/pesticide conc. (ng/g))
D2	0.03	0.84									
	4.6	99									
D5		0.04									
		4.85									
D6											
D7										0.07	

Table 2 (continued)

										7.44	
D8	0.23 40.0	0.57 68.0									13.3 121
D10	0.27 48.0										
D11		0.10 11.9									
D14					0.51 58.7				0.07 7.73		
D15											
D16								0.08 6.12	0.18 10.5	0.01 19.4	
D20			0.73 10.6		0.09 9.8						
D24	0.03 6.08			0.4 47.4	0.09 10.1	0.3 7.56					
D25	0.02 4.19		0.32 4.66	0.2 22.6							
D28	0.02 3.06			1.7 191		0.3 6.49	0.12 14.1	0.05 3.61			
D41			0.68 9.83				0.08 10.0				

more than 10 pollen samples within these days (Table S5). Methiocarb often had the highest PHQ values despite lower concentrations which thus contributed mainly to the high tPHQday values.

Discussion

To our knowledge, we present for the first time a full range pesticide analysis of daily pollen samples, collected over an entire season in one of the largest contiguous fruit-growing regions in Germany. Evaluation of the collected data from 102 consecutive days has resulted in some general conclusions. Almost 90% of the analyzed daily pollen samples in our study showed detectable concentrations of pesticide residues ranging from one to thirteen pesticides per day sample. The 29 detected pesticides were dominated by 51% fungicides and then 41% insecticides and less than 10% herbicides. This trend is corroborated by results shown in studies by Drummond et al. (2018) and Böhme et al. (2018), although they observed comparatively lower insecticide levels (10–25%) in their samples. Our observation can be further supported by the results of another German study examining pesticide use over several seasons at different farms in Germany (Bürger et al. 2012). They could show diverse pesticide utilization on every individual farm, even during the same specific season. However, all farms showed a continuous usage of minimum two fungicide and one herbicide treatments per season. Some farms also use one to three insecticides, depending on the type of cultivation. Most maximum concentrations in our study were measured during April (D1 to D17) and the first half of May (D18 to D32) as well as during the second part of June (D59 to D66) (Figs. 2 a and b and 3 a and b). Fungicides and insecticides were detected throughout the whole study from D1 to D102 (April to July), while the two detected herbicides (pendimethalin and dimethenamid) were present only from D43 to D81. The repeated occurrence of active substances during the study could be due to applications to different fields, changes in pollen availability in the landscape, and bees visiting other places that have pesticide exposure such as home gardens or roadside maintenance. Due to incorrect use or drift, pesticides can also find their way into other matrices as well. Another German study detected pesticide residues in surface water in an area where 41% of the landscape is used for crop cultivation. They detected pesticide residues in water samples over an entire year, with seasonal differences in pesticide concentrations between 0.05 and 14 µg/L (Müller et al. 2002). These results confirm that pesticides cannot only be found in pollen-producing plants during a flowering season, but, moreover they become spread across the environment and thus present a permanent risk. Three pesticides (diflubenzuron, fenoxycarb, and spirodiclofen) were classified as hazardous to bees, while at the same time diflubenzuron and fenoxycarb are not approved for the usage to blooming crops in Germany (Federal Office of Consumer

Protection and Food Safety 2020). Especially fenoxycarb is known to have negative effects on the bee brood; already a concentration of 50 ng/larva has been shown to induce significant damage (Czoppelt 1991; Aupinel et al. 2007). With a maximum value of 370 ng/g pollen fenoxycarb in our analyzed samples and a daily consumption of 9.5 mg bee bread per day by a nurse bee, the potential damage imposed on larvae may be low but should not be underestimated (Rortais et al. 2005). In comparison to this, Böhme et al. (2018) detected far lower concentrations of 6 ng fenoxycarb per pollen in their samples. The contamination with these pesticides could have been caused by accidental drift from other plants or another field or as a consequence of incorrect application (Pimentel 1995; de Jong et al. 2008; Lee et al. 2011). Frequently, pesticides were detected for time periods of around 10 consecutive days, exhibiting two or three maxima values, each of which several times higher compared to the previous highest concentration. This particular course was further verified by the simultaneous detection of tebuconazole and fluopyram, reaching their highest concentrations at 4500 ng/g pollen (Fig. 2). Comparatively lower pesticide concentrations were found in pollen samples from France at their maximum of 2020 ng tau-fluvalinate per g pollen (Chauzat et al. 2006). However, in our daily collected samples, maximum concentrations approached about 60% of the fenhexamid maximum level, as seen from the literature reporting a respective maximum at 7200 ng/g pollen. This was detected in a fruit-growing area in Southern Germany (Böhme et al. 2018) and was only about 5% compared to the maximum concentrations of up to 99,000 ng chlorothalonil per g pollen measured in the USA (Mullin et al. 2010; Stoner and Eitzer 2013). Typically, the maximum values in our present study were measured within the first 5 days of detection.

With respect to specific data, the data here does not indicate the occurrence acute toxic concentrations to honey bees for any of the detected pesticides. However, a general risk assessment should also include sublethal and synergistic effects of "pesticide cocktails" (Wade et al. 2019; Wernecke et al. 2019). The PHQmax values ranged between 0.09 and 170 within all observations. Fungicides and herbicides tend to show low PHQmax values between 0.08 and 55, whereas insecticides calculated higher PHQmax values to a maximum of 170 in 1-day sample. The tPHQday exceeded the relevant threshold of 50 in fifteen samples. A PHQ value of thiacloprid could be calculated in all of these samples, but with PHQ values below 10. Methiocarb showed the highest PHQ values up to 170 and, despite low pesticide concentrations, made the greatest contribution to the high PHQ values. Between D6 and D8, bees were consecutively exposed to tPHQday scores above 100 on each day, so they consumed over 500 tPHQ during this 4-day window. This is equivalent to 0.5% of the bees LD_{50} during a short 4-day window, with potentially serious implications for bee health. In comparison to the total pesticide concentrations in each daily sample, a connection between the total pesticide concentrations and the tPHQday values can only be shown in a few cases. It is notable that between D6 and D8, high tPHQday levels between 140 and 180 can be calculated, whereas the absolute pesticide concentrations only peak on days D9 to D11. However, the tPHQday values in this range are also above the relevant threshold of 50. In contrast, on D41 only a low total pesticide concentration of 270 ng/g could be measured, but the tPHQday value is over 165. With regard to which class contributes to the absolute pesticide concentration per day, it can be clearly seen that mainly fungicides followed by herbicides which are responsible for the highest pesticide concentrations. Insecticides only show a small contribution to the overall pesticide concentration (Fig. 4a). On the other hand, the class composition in the PHQ values clearly shows that insecticides in particular contribute to the high PHQ values in daily samples. In this particular case, we conclude that insecticides have a high influence on the tPHQday value, even if present at relatively low concentrations (Böhme et al. 2018; Favaro et al. 2019) (Fig. 4b, Table 2). The PHQ values presented in this study appeared lower compared to previously reported 500 to 4000 (McArt et al. 2017; Böhme et al. 2018) and even higher than 40,000, as reported mainly in other studies undertaken in the USA, where more stringent plant protection management is commonly executed (Stoner and Eitzer 2013; Favaro et al. 2019). Not included in general risk assessments of pesticide residues are other pollinators, especially wild bees. These pollinators have a small foraging range and often a short foraging period of only several days. Such insect pollinators are (i) less likely to escape from a treated field and (ii) their brood is predominantly reared directly on stored pollen contaminated by a localized pesticide mixture. This particular condition may provoke a significantly negative impact on the diversity of native pollinators (Tuell and Isaacs 2010; Mallinger et al. 2015; Park et al. 2015).

The occurrence of highest pesticide concentrations in only a few individual daily pollen samples may be rationalized by the assumption that in pooled pollen samples, maximum concentrations were being mitigated by dilution. For instance, fluopyram and tebuconazole which were detected together from D7 to D16 would have been expected at 1000 ng/g pollen in a respective 10-day sample pool but were only showing at 400 ng/g pollen in a pooled monthly sample (Fig. 5, Table S6). The specific dilution effect we have shown here is likely to be of general significance with respect to the evaluation of pesticide concentrations in all pooled samples. Hence, pooled pollen samples may contribute to a general underestimation of the threat to which honey bees are exposed during particular single day by a factor of between fourfold up to tenfold. This kind of potentially erroneous data evaluation turns out to be particularly important because specific maximum concentrations were found to be comparably low within the immediate surroundings of the hive. In various other

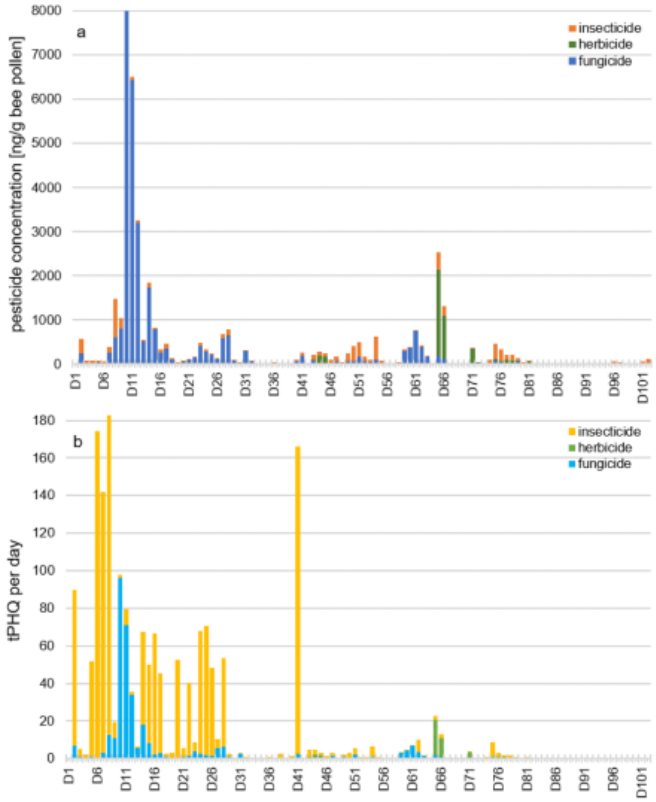

Fig. 4 Bar chart with a day-to-day progression of (**a**) the total pesticide concentration per day and (**b**) the tPHQday values (divided into fungicide, herbicide, and insecticide class)

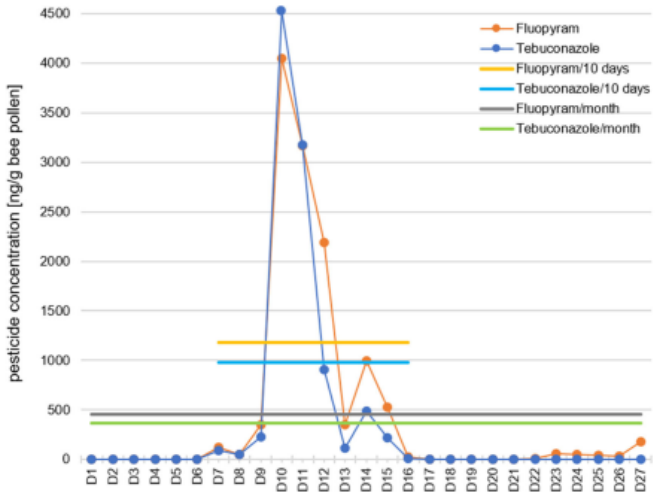

Fig. 5 Pesticide concentrations of fluopyram and tebuconazole per day or on averaged 10 days or a full month

situations, pesticide loads detected in daily pollen samples may also lead to the underestimation of corresponding threats to honey bees and other insects. Further extensive analytical investigations are required, across different agricultural regions, with a focus on the real daily exposure of honey bees and other pollinators to the wide range of various applied pesticide cocktails (Ostiguy et al. 2019).

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-020-12318-2.

Acknowledgments The authors are thankful to the volunteer beekeeper for collecting bee pollen for over 4 months. Also, thanks to Dr. Raghdan Alkattea for palynological analysis of exemplary pollen samples. Furthermore, we want to thank Evin Erenler for supporting the graphical representation and evaluation.

Authors' information Not applicable.

Authors' contributions Carolin Friedle (conceptualization, data curation, methodology, analysis, resources, visualization, writing (original draft), writing (review and editing)); Klaus Wallner (conceptualization, data curation, methodology, funding acquisition, supervision, writing (review and editing)); Peter Rosenkranz (conceptualization, funding acquisition, writing (review and editing)); Dieter Martens (data curation, methodology, analysis, writing (review and editing)); and Walter Vetter (conceptualization, data curation, supervision, writing (review and editing)).

Funding Open Access funding enabled and organized by Projekt DEAL. This study was supported by the Ministry of Rural Areas and Consumer Protection Baden-Wuerttemberg.

Data availability The authors confirm the data generated or analyzed during this study are included in this published article and its supplementary information files. All datasets used and analyzed during the current study is also available from the corresponding author on reasonable request.

Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

Ethics approval The authors confirm that ethical standards were addressed.

Consent to participate The authors confirm the volunteer's declaration of consent.

Consent to publish The authors confirm the volunteer's consent for publication. The data (including the map) generated by the Environmental Information System (UIS) of the State Institute for Environment Baden-Wuerttemberg have be given permission to be published.

References

Aktar MW, Sengupta D, Chowdhury A (2009) Impact of pesticides use in agriculture: their benefits and hazards. Interdiscip Toxicol 2(1):1–12. https://doi.org/10.2478/v10102-009-0001-7

Anastassiades M, Lehotay SJ, Štajnbaher D, Schenck FJ (2003) Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. J AOAC Int 86(2):412–431. https://doi.org/10.1093/jaoac/86.2.412

Anderson KE, Carroll MJ, Sheehan T, Lanan MC, Mott BM, Maes P, Corby-Harris V (2014) Hive-stored pollen of honey bees: many lines of evidence are consistent with pollen preservation, not nutrient conversion. Mol Ecol 23(23):5904–5917. https://doi.org/10.1111/mec.12966

Attallah ER, Amer ME, Gomaa AM, El Gohary AA (2012) QuEChERS analytical method for determination of 93 pesticide residues in apples and potatoes using LC-MS/MS. J Appl Sci Res 8(12):5690–5697

Aupinel P, Fortini D, Michaud B, Marolleau F, Tasei J-N, Odoux J-F (2007) Toxicity of dimethoate and fenoxycarb to honey bee brood (*Apis mellifera*), using a new in vitro standardized feeding method. Pest Manag Sci 63(11):1090–1094. https://doi.org/10.1002/ps.1446

Böhme F, Bischoff G, Zebitz CPW, Rosenkranz P, Wallner K (2018) Pesticide residue survey of pollen loads collected by honeybees (*Apis mellifera*) in daily intervals at three agricultural sites in South Germany. PLoS One 13(7):e0199995. https://doi.org/10.1371/journal.pone.0199995

Böhme F, Bischoff G, Zebitz CPW, Rosenkranz P, Wallner K (2019) From field to food II – will pesticide-contaminated pollen diet lead to a contamination of worker jelly? J Apic Res 86(7):1–8. https://doi.org/10.1080/00218839.2019.1614727

Botías C, David A, Horwood J, Abdul-Sada A, Nicholls E, Hill E, Goulson D (2015) Neonicotinoid residues in wildflowers, a potential route of chronic exposure for bees. Environ Sci Technol 49(21):12731–12740. https://doi.org/10.1021/acs.est.5b03459

Bürger J, de MF, Gerowitt B (2012) Influence of cropping system factors on pesticide use intensity – a multivariate analysis of on-farm data in North East Germany. Eur J Agron 40:54–63. https://doi.org/10.1016/j.eja.2012.02.008

Carroll MJ, Brown N, Goodall C, Downs AM, Sheenan TH, Anderson KE (2017) Honey bees preferentially consume freshly-stored pollen. PLoS One 12(4):e0175933. https://doi.org/10.1371/journal.pone.0175933

Chauzat M-P, Faucon J-P, Martel A-C, Lachaize J, Cougoule N, Aubert M (2006) A survey of pesticide residues in pollen loads collected by honey bees in France. 99(2):253–262. https://doi.org/10.1603/0022-0493-99.2.253

Codling G, Naggar YA, Giesy JP, Robertson AJ (2018) Neonicotinoid insecticides in pollen, honey and adult bees in colonies of the European honey bee (*Apis mellifera* L.) in Egypt. Ecotoxicology (London, England) 27(2):122–131. https://doi.org/10.1007/s10646-017-1876-2

Cridge AG, Leask MP, Duncan EJ, Dearden PK (2015) What do studies of insect polyphenisms tell us about nutritionally-triggered

epigenomic changes and their consequences? Nutrients 7(3):1787–1797. https://doi.org/10.3390/nu7031787

Czoppelt C (1991) Toxicity measurements with the juvenoid fenoxycarb on bee larvae in the in vitro breeding test. (Toxizitätsmessungen mit dem Juvenoid Fenoxycarb an Bienenlarven im in vitro Aufzuchttest). Apidologie 22(4):457–459

DeGrandi-Hoffman G, Eckholm BJ, Huang MH (2013) A comparison of bee bread made by Africanized and European honey bees (*Apis mellifera*) and its effects on hemolymph protein titers. Apidologie 44(1):52–63. https://doi.org/10.1007/s13592-012-0154-9

Detroy BF, Harp ER (1976) Pollen traps - trapping pollen from honey bee colonies. Agricultural Research Service. United States Department of Agriculture. Production Research Report No. 163

Drummond FA, Ballman ES, Eitzer BD, Du Clos B, Dill J (2018) Exposure of honey bee (*Apis mellifera* L.) colonies to pesticides in pollen, a statewide assessment in Maine. Environ Entomol 47(2): 378–387. https://doi.org/10.1093/ee/nvy023

Favaro R, Bauer LM, Rossi M, D'Ambrosio L, Bucher E, Angeli S (2019) Botanical origin of pesticide residues in pollen loads collected by honeybees during and after apple bloom. Front Physiol 10:1069. https://doi.org/10.3389/fphys.2019.01069

Federal Office of Consumer Protection and Food Safety (2020) Online data base on approved plant protection products. https://apps2.bvl.bund.de/psm/jsp/index.jsp. Accessed April 2020

German Bee Monitoring (2014–2019) German bee monitoring (Deutsches Bienenmonitoring). https://bienenmonitoring.uni-hohenheim.de/. Accessed 22 Jun 2020

Info Service of Agricultural - Nutrition and Rural Areas (2018) Agriculture in Baden-Wuerttemberg- facts and figures. (Landwirtschaft in Baden-Württemberg - Daten und Fakten). https://www.landwirtschaft-bw.info/pb/Lde/3650826_3651462_5405915_5378885_5378985_5378998_5422965. Accessed April 2020

de Jong FMW, de Snoo GR, van de Zande JC (2008) Estimated nationwide effects of pesticide spray drift on terrestrial habitats in the Netherlands. J Environ Manag 86(4):721–730. https://doi.org/10.1016/j.jenvman.2006.12.031

Keller I, Fluri P, Imdorf A (2005) Pollen nutrition and colony development in honey bees—Part II. Bee World 86(2):27–34. https://doi.org/10.1080/0005772X.2005.11099650

Kevan PG, Baker HG (1983) Insects as flower visitors and pollinators. Annu Rev Entomol 28(1):407–453. https://doi.org/10.1146/annurev.en.28.010183.002203

Klein S, Pasquaretta C, He XJ, Perry C, Søvik E, Devaud J-M, Barron AB, Lihoreau M (2019) Honey bees increase their foraging performance and frequency of pollen trips through experience. Sci Rep 9(1):6778. https://doi.org/10.1038/s41598-019-42677-x

Lee S-J, Mehler L, Beckman J, Diebolt-Brown B, Prado J, Lackovic M, Waltz J, Mulay P, Schwartz A, Mitchell Y, Moraga-McHaley S, Gergely R, Calvert GM (2011) Acute pesticide illnesses associated with off-target pesticide drift from agricultural applications: 11 States, 1998–2006. Environ Health Perspect 119(8):1162–1169. https://doi.org/10.1289/ehp.1002843

Lindauer M (1952) A contribution to the question of the division of labor in the bee state. (Ein Beitrag zur Frage der Arbeitsteilung im Bienenstaat). Z Vergl Physiol 34(4):299–345. https://doi.org/10.1007/BF00298048

Mallinger RE, Werts P, Gratton C (2015) Pesticide use within a pollinator-dependent crop has negative effects on the abundance and species richness of sweat bees, Lasioglossum spp., and on bumble bee colony growth. J Insect Conserv 19(5):999–1010. https://doi.org/10.1007/s10841-015-9816-z

McArt SH, Fersch AA, Milano NJ, Truitt LL, Böröczky K (2017) High pesticide risk to honey bees despite low focal crop pollen collection during pollination of a mass blooming crop. Sci Rep 7:46554. https://doi.org/10.1038/srep46554

McGregor SE (1976) Insect pollination of cultivated crop plants, 1st edn. United Department of Agriculture, Washington D.C

Müller K, Bach M, Hartmann H, Spiteller M, Frede H-G (2002) Point- and nonpoint-source pesticide contamination in the Zwester Ohm catchment, Germany. J Environ Qual 31(1):309–318. https://doi.org/10.2134/jeq2002.3090

Mullin CA, Frazier M, Frazier JL, Ashcraft S, Simonds R, Vanengelsdorp D, Pettis JS (2010) High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. PLoS One 5(3):e9754. https://doi.org/10.1371/journal.pone.0009754

Nagai T, Nagashima T, Suzuki N, Inoue R (2005) Antioxidant activity and angiotensin I-converting enzyme inhibition by enzymatic hydrolysates from bee bread(60c):133–138. doi: https://doi.org/10.1515/znc-2005-1-224

Ostiguy N, Drummond FA, Aronstein K, Eitzer B, Ellis JD, Spivak M, Sheppard WS (2019) Honey bee exposure to pesticides: a four-year nationwide study. Insects 10(1). https://doi.org/10.3390/insects10010013

Park MG, Blitzer EJ, Gibbs J, Losey JE, Danforth BN (2015) Negative effects of pesticides on wild bee communities can be buffered by landscape context. Proc Biol Sci 282(1809):20150299. https://doi.org/10.1098/rspb.2015.0299

Pesticides Properties DataBase - PPDB (2020) A to Z list of pesticide active ingredients. University of Hertfordshire, Available from https://sitem.herts.ac.uk/aeru/ppdb/en/atoz.htm

Pimentel D (1995) Amounts of pesticides reaching target pests: environmental impacts and ethics. J Agric Environ Ethics 8:17–29

Rortais A, Arnold G, Halm M-P, Touffet-Briens F (2005) Modes of honeybees exposure to systemic insecticides: estimated amounts of contaminated pollen and nectar consumed by different categories of bees. Apidologie 36(1):71–83. https://doi.org/10.1051/apido:2004071

Roßberg D, Harzer U (2015) Surveys on the use of pesticides in apple growing areas. Erhebungen zur Anwendung von Pflanzenschutzmitteln im Apfelanbau). J Crops(67 (3)). doi: https://doi.org/10.5073/JFK.2015.03.01

Statistical Service Office Baden-Wuerttemberg (2017) Apples dominate fruit growing in Baden-Wuerttemberg. (Äpfel dominieren den Obstanbau in Baden-Württemberg). http://www.statistik-bw.de/Presse/Pressemitteilung/2017/187. Accessed April 2020

Statistical Service Office Baden-Wuerttemberg (2020) Population, area and population density since 1961. Population, area and population density since 1961 - Lake Constance region. (Bevölkerung, Gebiet und Bevölkerungsdichte - Gemeindengebiet, Bevölkerung und Bevölkerungsdichte seit 1961 - Landkreis Bodenseekreis). https://www.statistik-bw.de/BevoelkGebiet/Bevoelkerung/01515020.tab?R=KR435. Accessed April 2020

Steffan-Dewenter I, Kuhn A (2003) Honeybee foraging in differentially structured landscapes. Proc Biol Sci 270(1515):569–575. https://doi.org/10.1098/rspb.2002.2292

Stoner KA, Eitzer BD (2013) Using a hazard quotient to evaluate pesticide residues detected in pollen trapped from honey bees (*Apis mellifera*) in Connecticut. PLoS One 8(10):e77550. https://doi.org/10.1371/journal.pone.0077550

The Scottish Government (2018) Pesticide Usage in Scotland: Soft Fruit Crops 2018 https://www.gov.scot/publications/pesticide-usage-scotland-soft-fruit-crops-2018/

Tosi S, Costa C, Vesco U, Quaglia G, Guido G (2018) A 3-year survey of Italian honey bee-collected pollen reveals widespread contamination by agricultural pesticides. Sci Total Environ 615:208–218. https://doi.org/10.1016/j.scitotenv.2017.09.226

Traynor KS, Pettis JS, Tarpy DR, Mullin CA, Frazier JL, Frazier M, Vanengelsdorp D (2016) In-hive pesticide exposome: assessing risks to migratory honey bees from in-hive pesticide contamination in the Eastern United States. Sci Rep 6:33207. https://doi.org/10.1038/srep33207

Tuell JK, Isaacs R (2010) Community and species-specific responses of wild bees to insect pest control programs applied to a pollinator-dependent crop. J Econ Entomol 103(3):668–675. https://doi.org/10.1603/ec09314

Wade A, Lin C-H, Kurkul C, Regan ER, Johnson RM (2019) Combined toxicity of insecticides and fungicides applied to California almond orchards to honey bee larvae and adults. Insects 10(1). https://doi.org/10.3390/insects10010020

Wallner K (2012) Not in the bloom. (Nicht in die Blüte). German bee J 9/2012:18–19

Wernecke A, Frommberger M, Forster R, Pistorius J (2019) Lethal effects of various tank mixtures including insecticides, fungicides and fertilizers on honey bees under laboratory, semi-field and field conditions. J Consum Prot Food Saf 14(3):239–249. https://doi.org/10.1007/s00003-019-01233-5

Willmer P (2011) Pollination and floral ecology. Princeton University Press, New Jersey

3.2 Changes of microorganism composition in fresh and stored bee pollen from Southern Germany

Publiziert in *Environmental Science and Pollution Research:*

Abstract

Analysis of plant pollen can provide valuable insights into the existing spectrum of microorganisms in the environment. When harvesting bee-collected pollen as a dietary supplement for human consumption, timely preservation of the freshly collected pollen is fundamental for product quality. Environmental microorganisms contained in freshly collected pollen can lead to spoilage by degradation of pollen components. In this study, freshly collected bee pollen was sampled at different locations and stored under various storage conditions to examine the hypothesis, that storage conditions may have an effect on the composition of microorganisms in pollen samples. The samples were analyzed using 16S and 18S amplicon sequencing and characterized by palynological analysis. Interestingly, the bacterial communities between pollen samples from different locations varied only slightly, whereas for fungal community compositions this effect was substantially increased. Further, we noticed that fungal communities in pollen are particularly sensitive to storage conditions. The fungal genera proportion *Cladosporium* and *Mycosphaerella* decreased, while *Zygosaccharomyces* and *Aspergillus* increased during storage. *Aspergillus* and *Zygosaccharomyces* fractions increased during storage at 30 °C, which could negatively impact the pollen quality if it is used as a dietary supplement.

Friedle, C.; D'Alvise, P.; Schweikert, K.; Wallner, K.; Hasselmann, M. (2021): Changes of microorganism composition in fresh and stored bee pollen from Southern Germany. In: *Environ Sci Pollut Res*. DOI: 10.1007/s11356-021-13932-4.

Environmental Science and Pollution Research
https://doi.org/10.1007/s11356-021-13932-4

RESEARCH ARTICLE

Changes of microorganism composition in fresh and stored bee pollen from Southern Germany

Carolin Friedle [1,2] · Paul D'Alvise [2] · Karsten Schweikert [3] · Klaus Wallner [1] · Martin Hasselmann [2]

Received: 22 December 2020 / Accepted: 9 April 2021

Abstract
Analysis of plant pollen can provide valuable insights into the existing spectrum of microorganisms in the environment. When harvesting bee-collected pollen as a dietary supplement for human consumption, timely preservation of the freshly collected pollen is fundamental for product quality. Environmental microorganisms contained in freshly collected pollen can lead to spoilage by degradation of pollen components. In this study, freshly collected bee pollen was sampled at different locations and stored under various storage conditions to examine the hypothesis that storage conditions may have an effect on the composition of microorganisms in pollen samples. The samples were analyzed using 16S and 18S amplicon sequencing and characterized by palynological analysis. Interestingly, the bacterial communities between pollen samples from different locations varied only slightly, whereas for fungal community compositions, this effect was substantially increased. Further, we noticed that fungal communities in pollen are particularly sensitive to storage conditions. The fungal genera proportion *Cladosporium* and *Mycosphaerella* decreased, while *Zygosaccharomyces* and *Aspergillus* increased during storage. *Aspergillus* and *Zygosaccharomyces* fractions increased during storage at 30 °C, which could negatively impact the pollen quality if it is used as a dietary supplement.

Keywords Microorganism composition · Bee pollen · Pollen quality · Pollen spoilage

Introduction

The existing spectrum of microorganisms in the environment can be revealed in pollen samples from plants (Anderson et al. 2013; Manirajan et al. 2019). Pollen, the male gametophyte of gymnosperms and angiosperms, represents the main protein and lipid source for honey bees (*Apis mellifera*). It provides a full spectrum of not only nutrients, mainly amino acids and lipids, but also carbohydrates, minerals, vitamins, and enzymes (Feás et al. 2012; Pascoal et al. 2014; Avni et al. 2014; Kostić et al. 2015). Bee-collected pollen consists of pollen grains from flowering plants (including bushes and trees), as well as nectar and salivary secretion from the bees. It is collected by foraging honey bees and transported to the hive in the pollen baskets known as corbicula on their hind legs (Kevan and Baker 1983; Willmer 2011). After storage and microbe-mediated maturation in honeycomb cells, pollen is called "bee bread" and is consumed by nurse bees to produce protein-rich larval food supported by secretions of specialized glands (Lindauer 1952; Cridge et al. 2015). Bee pollen is perceived as a useful dietary supplement for humans, as it provides many necessary nutrients, especially a high amount of protein (± 20%) including essential amino acids like leucine, isoleucine, and valine, depending on the botanical origin (Paramás et al. 2005; Carpes et al. 2009; Martins et al. 2011; Feás et al. 2012; Nicolson and Human 2013; Taha et al. 2019).

The nutrient composition and microbiological quality of bee pollen depend strongly on its geographic and botanical origin, the weather at the time of collection, as well as on the post-harvest processing procedure by the bee keeper (Hani et al. 2012; Nogueira et al. 2012; Corby-Harris et al. 2014; De-Melo et al. 2015, 2016; Dinkov 2016;

Responsible editor: Diane Purchase

✉ Carolin Friedle
carolin_friedle@uni-hohenheim.de

[1] Apicultural State Institute, University of Hohenheim, Stuttgart, Germany

[2] Institute of Animal Science, Department of Livestock Population Genomics, University of Hohenheim, Stuttgart, Germany

[3] Core Facility Hohenheim and Institute of Economics, University of Hohenheim, Stuttgart, Germany

Published online: 23 April 2021

Disayathanoowat et al. 2020). When gathering of the pollen is not followed by drying or another processing step, growth of microorganisms can compromise pollen quality, leading to negative side effects like fermentation or mycotoxin production (González et al. 2005; Hani et al. 2012; Fatrcová-Šramková et al. 2016). The taxonomical identity of the identified microorganisms in fresh bee pollen suggests that the initial microbial community composition is rather arbitrary and may change in composition to more opportunistic microorganisms with unwanted characteristics that grow well under warm storage conditions (González et al. 2005; Hani et al. 2012). Following the hypothesis that storage conditions may impact the composition of microorganisms, resulting in the spoilage of pollen, we measured the differences in the microorganism composition of freshly harvested bee pollen and compared it with the same pollen after storage under different temperature treatments. This experimental approach aimed to identify the most relevant microorganisms that cause spoilage and identify the most favorable storage condition for preserving pollen quality during and after the harvest.

Previous studies have used cultivation-dependent methods, e.g., bacterial and fungal colony counts on agar plates (Bonvehí and Jordà 1997; González et al. 2005; Estevinho et al. 2012; Feás et al. 2012; Bárbara et al. 2015; De-Melo et al. 2015; Dinkov 2018), and cultivation-independent methods, such as 16S-rRNA amplicon sequencing to characterize the microbial composition in fresh, dried of frozen bee pollen to evaluate the risk of microbial hazards (Anderson et al. 2014; Corby-Harris et al. 2014; Mauriello et al. 2017; Moreno Andrade et al. 2018).

Recently, Disayathanoowat et al. (2020) analyzed the abundances of bacterial and fungal communities in fresh bee pollen and in "bee bread" that was stored for 72 h within the hive using amplicon sequencing and plate counting. They showed that abundance of bacteria was declining, while the fungal population remained at stable numbers during in-hive storage. However, to our knowledge, there is no information available about changes in bacterial and fungal communities in pollen being stored outside of the hive under defined conditions. Furthermore, taxonomic information about changes in the microbial communities of stored pollen are missing. Consequently, the present study was designed to assess the qualitative changes by next-generation sequencing of 16S and 18S PCR-amplicons in the microbial communities of pollen. Samples were stored under different conditions, to simulate how the composition of the microorganisms can be affected by wrong processing or non-harvesting of pollen.

Materials and methods

Sampling Bee pollen was collected on 1 day in the month of June in 2 consecutive years at three different locations: Baden-Wuerttemberg, Southern Germany (Hohenheim in 2018 (H), Forbach in 2019 (F), and Nuertingen in 2019 (N)) (Figure S1). To minimize the presence of any potential microorganism on the pollen traps before, the traps were cleaned intensively with 70% ethanol and installed in front of one hive per site to collect pollen loads from returning honey bees (*Apis mellifera*) (Detroy and Harp 1976). The freshly collected pollen was divided at the same day of collection and allocated to four different groups "fresh," "cold," "room temperature," and "warm." Condition "cold" simulated a storage in the refrigerator and "room temperature" a storage without refrigeration after harvesting. The condition "warm" simulated unharvested pollen in the trap or pollen was left on environmental conditions of a hot summer day. The samples representing each group were further divided into triplicate samples of 3 g and filled into 2 mL tubes (VWR International, Bruchsal, Germany). Group "fresh" was stored immediately at − 80 °C until extraction, while the other groups were incubated at different temperatures for 7 days (168 h) and thereafter stored at − 80 °C until extraction. Group "cold" was incubated at cold temperatures (4 °C), group "room temperature" was incubated at 25 °C, and group "warm" was incubated at 30 °C and 75% humidity (humidity was adjusted with a saturated sodium chloride solution). All samples were incubated with open lids in humidity chambers (Figure S2) and samples of the groups "warm" and "room temperature" were further incubated in a warming cabinet (Binder, Tuttlingen, Germany) and samples of group "cold" were placed in a refrigerator (Siemens, Munich, Germany).

Pollen samples in Hohenheim were collected directly by the authors; samples from Forbach and Nuertingen were collected with the help of voluntary beekeepers. All samples were received from privately owned bee colonies, so no exact grid references are given, and no permits were needed for this study.

Palynological analysis A palynological analysis of all pooled samples of each location was performed. All pollen samples were mechanically homogenized by using a mortar, followed by weighing 100 mg homogenized pollen in a 50-mL tube (Buddeberg, Mannheim, Germany) containing 10 mL demineralized water and a drop of dish soap (Friedle et al. 2021). For each sample, 500 pollen grains were determined. Pollen morphology was identified using a light microscope (10 × 40; VWR International, Bruchsal, Germany).

DNA extraction To analyze the microbial community, DNA was extracted from all 36 bee pollen samples using a TRIzol protocol (D'Alvise et al. 2018; Seeburger et al. 2020). An aliquot of 50 mg pollen was weighted in to a 2 mL lysis tube with 50 µL 0.1 mm glass/zirconia beads and 500 µL TRIzol (Invitrogen, ThermoFisher Scientific, Schwerte, Germany). The samples were homogenized in a bead beater (FastPrep-

Table 1 Classification of pollen diversity in the samples

Classification	Hohenheim 2018	Forbach 2019	Nuertingen 2019
Aceraceae_Acer	-	42%	-
Boraginaceae_Phacelia	19%	-	-
Brassicaceae_Sinapis-T	10%	-	-
Malvaceae_Tilia	8%	-	8%
Oleaceae_Ligustrum	-	0.2%	16%
Plantaginaceae_Plantago	14%	3%	5%
Ranunculaceae	-	12%	-
Rosaceae_Aruncus dioicus	-	0.4%	42%
Rosaceae_Filipendula	10%	-	2%
Sapindacaea_Aesculus	0.4%	10%	-
Others	38%	33%	28%

24, MP Biomedicals, Eschwege, Germany) at 5.5 ms^{-1} for 50 s and incubated for 5 min at room temperature (RT). After adding 100 µL chloroform, shaking for 15 s, and further incubation for 5 min at RT, two phases were separated by 15 min centrifugation at 12,000×*g* and 4 °C. The aqueous phase was transferred to another tube for RNA extraction, which can be used for further experiments. To the other phase, 250 µL back extraction buffer (4 M guanidine thio-cyanate, 50 mM sodium citrate, 1 M TRIS) were added and extracted by shaking for 15 s. After 10 min incubation at RT and centrifugation (as before), the aqueous supernatant was transferred to a new tube and precipitated by mixing with 200 µL isopropanol, followed by centrifugation (as before). The supernatant was discarded, and 500 µL 75% ethanol were added to wash the sediment. After short centrifugation (5 min; 2000×*g*; 4 °C), the supernatant was removed and the sediment dried for 10 min at RT. An aliquot of 50 µL 8 mM NaOH were added to redissolve the sediment and the solution was centrifuged for 10 min at 12,000×*g* at RT to remove membrane lipids. The supernatant was transferred to a new tube and mixed with 4.25 µL 0.1 M HEPES and 0.5 µL RNAse A (10 mg ml^{-1} Amresco). The DNA extracts were then incubated for 1 h at 37 °C and stored at −20 °C until analysis. The DNA concentrations were determined using Qubit fluorometer (Thermo Fisher Scientific, Schwerte, Germany) and showed concentrations < 1 ng/µL. Some of the DNA extracts requiring additional purification were thereafter precipitated with absolute ethanol; pH was adjusted to 5.5 with 5 µL 3 M sodium acetate solution, then the sample was mixed with 125 µL cold ethanol (absolute). After incubation (15 min at 4 °C) and centrifugation (20 min; 17,000×*g*; 4 °C), the supernatant was removed, the sediment was dried for 5 min at RT and dissolved in 20 µL nuclease-free water.

PCR and amplicon sequencing Amplicons (using a volume of 10 µL in a 20 ng template) of the V3–V4 region in the bacterial 16S-rRNA-gene and amplicons of the ITS1 region in the fungal 18S-rRNA-gene were generated and Illumina-sequenced in 2018 by Eurofins Genomics (Ebersberg, Germany) ("Dataset 1"). The PCR conditions followed by library preparation and sequencing were described previously (D'Alvise et al. 2018). Primers for the V3–V4 region of the 16S-rRNA gene were 5′-TACGGGAGGCAGCAG (F) and 5′-CCAGGGTATCTAATCC (R) (Turner et al. 1999; Kisand and Wikner 2003). Primers for the fungal ITS1 region were 5′-GGAAGTAAAAGTCGTAACAAGG (F) and 5′-GCTGCGTTCTTCATCGATGC (R) (White et al. 1990). Amplicons from samples in 2019 were Illumina-sequenced by StarSEQ (Mainz, Germany) ("Dataset 2"). Primers for the V3–V4 region of the 16S-rRNA gene were 5′-CCTACGGGAGGCAGCAG (F) and 5′-GGACTACNNGGGTATCTAAT (R) (Klindworth et al. 2013). Primers for the fungal

Table 2 Sequences and OTUs within the raw data and after filtering with IMNGS and QIIME2

		Dataset 1 2018 (H)		Dataset 2 2019 (F, N)	
		Bacteria	Fungi	Bacteria	Fungi
Raw data	Sequences	164,508	1,013,947	108,399	2,893,070
	OTUs	453	1093	2215	5763
IMNGS/QIIME2	OTUs	56	131	33	1998
	Mean OTUs/sample	40.2	23.2	21.8	357.7

Table 3 Bacterial phyla composition with bacterial genera calculated with total reads in all samples (Dataset 2)

Actinobacteria (3%)	*Arthrobacter*
Bacteroidetes (4%)	*Apibacter*
	Bacteroides
	Chryseobacterium
	Epilithonimonas
	Flavobacterium
	Pedobacter
Cyanobacteria (0.1%)	*Cyanobium*
Firmicutes (44%)	*Fusicatenibacter*
	Lactobacillus
	Lactococcus
	Staphylococcus
Proteobacteria (48%)	*Acinetobacter*
	Actibacterium
	Arsenophonus
	Batronella
	Bradyrhizobium
	Carnimonas
	Citrobacter
	Duganella
	Erwinia
	Escherichia
	Frischella
	Gilliamella
	Gluconacetobacter
	Halotalea
	Massilia
	Neokomagataea
	Pantoea
	Pectobacterium
	Phaseolibacter
	Pseudomonas
	Rickettsia
	Rosenbergiella
	Saccharibacter
	Serratia
	Snodgrassella
	Sphingomonas

ITS1 region were 5′-CTTGGTCATTTAGAGGAAGTAA (F) and 5′-GCTGCGTTCTTCATCGATGC (R) (White et al. 1990; Gardes and Bruns 1993), an obviously modified and improved set compared to the one from Eurofins Genomics. For all primer sets, efficacies were reported to be high and evaluated by the standardized procedures of the companies.

The clean sequencing reads from the bacterial 16-rRNA-gene, as provided after quality control and trimming by the sequencing company, were analyzed on the IMNGS server platform (Lagkouvardos et al. 2016). Quality filtering showed %Q30 values of about 90% of the expected amplicon sizes. Analyses were performed without further trimming using a total abundance threshold of 0.1%, and the reads were binned on a 1% difference criterium (Edgar 2013; Lagkouvardos et al. 2016). Sequences from the fungal 18S-rRNA-gene were analyzed by StarSEQ using the QIIME2 platform. The taxonomic classification of the representative OTU sequences were controlled and refined by BLAST-searches against reference material in the NCBI database (https://blast.ncbi.nlm.nih.gov).

Statistical analysis Differences in the relative abundances of bacterial and fungal OTUs between locations and treatments were analyzed using an ANOVA-type generalized linear model (GLM). Since the analyzed relative abundances are bounded between zero and one, the data are generated by beta distribution (Ferrari and Cribari-Neto 2004). The model was estimated using the betareg package (Cribari-Neto and Zeileis 2010). To identify significant differences in the microbial communities between the different locations and storage conditions, a single-step multiple comparison test (Tukey's test) was performed. The reported p-values were corrected for multiple testing. All statistical analyses were performed using R version 3.6.2. with a significance level of $p = 0.05$.

Results

Characterization of pollen diversity

The palynological analysis showed, as expected, differences in pollen diversity at each location sites. At location H, pollen composition showed a high variety with 19% *Phacelia sp*., 14% *Plantago sp*., 10% *Filipendula sp*. and *Sinapsis-type*. Location N predominantly showed pollen of 42% *Aruncus sp*. and 16% *Ligustrum sp*., followed by 8% *Tilia sp*. Location F was dominated by 42% *Acer sp*. followed by 12% pollen from *Ranunculacaea* and 10% *Aesculus sp*. (Table 1, Table S1).

Quantitative data of amplicon sequencing

The total bacterial and fungal sequences and the total number of operational taxonomic units (OTUs) from 16S (bacteria) and 18S (fungi) before and after IMNGS/QIIME2 analyzing are shown in Table 2. After filtering (> 0.1%) and binning, 38 different bacteria genera and 33 different fungal genera were obtained, and their relative abundances were calculated (Table S2 and Table S3). A cut-off of 10% was performed for statistical analysis to obtain the most abundant bacteria and fungi (Table S4 and Table S5).

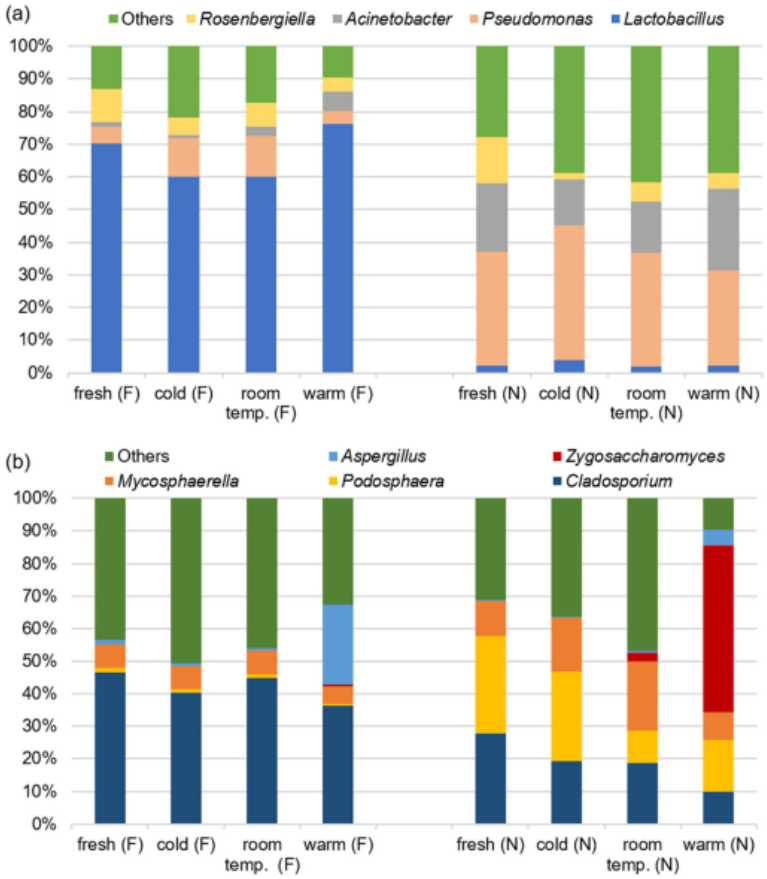

Fig. 1 Stack bar chart, showing the composition of bacterial (**a**) and fungal (**b**) communities of Dataset 2 (F and N 2019) (filtered on minimum of 10% average) in fresh and stored bee pollen

Differences between Dataset 1 and Dataset 2

Given the fact that for technical reasons the samples of 2018 (Dataset 1, H) and of 2019 (Dataset 2, F and N) were processed by different companies, a stringent comparison between both is not recommended. In particular, we noticed a high deviation in the fungal composition between Dataset 1 and Dataset 2 (Table 2), because primers with different binding specifications were used by the sequencing companies for the fungal ITS1 region. Analyses from Dataset 1 provided only data from *Ascomycota*, likely as a result of using non-fungi-specific primers (see "PCR and amplicon sequencing"). Consequently, the fungal composition from Dataset 1 consists of only three different genera in the samples and probably the full spectrum of fungal diversity in the pollen samples was not revealed in Dataset 1. Therefore, results from Dataset 1 were removed and all subsequent statistical analysis was performed only with Dataset 2. For the sake of completeness, the results of Dataset 1 are listed additionally in the supplemental material (Table S6, Table S7, and Figure S3).

Microbial diversity in fresh and stored bee pollen

The most abundant bacterial phyla in all samples were *Proteobacteria* (48%) and *Firmicutes* (44%), followed by *Bacteroidetes* (4%), *Actinobacteria* (3%), and *Cyanobacteria* (0.1%) in each of which between one and 26 different bacterial genera have been identified (Table 3). The main bacterial genera in Dataset 2 were *Lactobacillus* (2–76%), *Pseudomonas* (5–42%), and *Acinetobacter* (1–25%) (Fig. 1a) (Table S4). The percentage of *Acinetobacter* was higher in pollen after warm storage than in fresh pollen, while *Pseudomonas* and *Rosenbergiella* were less abundant after warm storage than in fresh pollen (Fig. 2). However, only a significant difference between storage conditions "room temperature – warm" could be found for *Lactobacillus* (GLM; $p = 0.039$; Tukey; $p = 0.043$). In

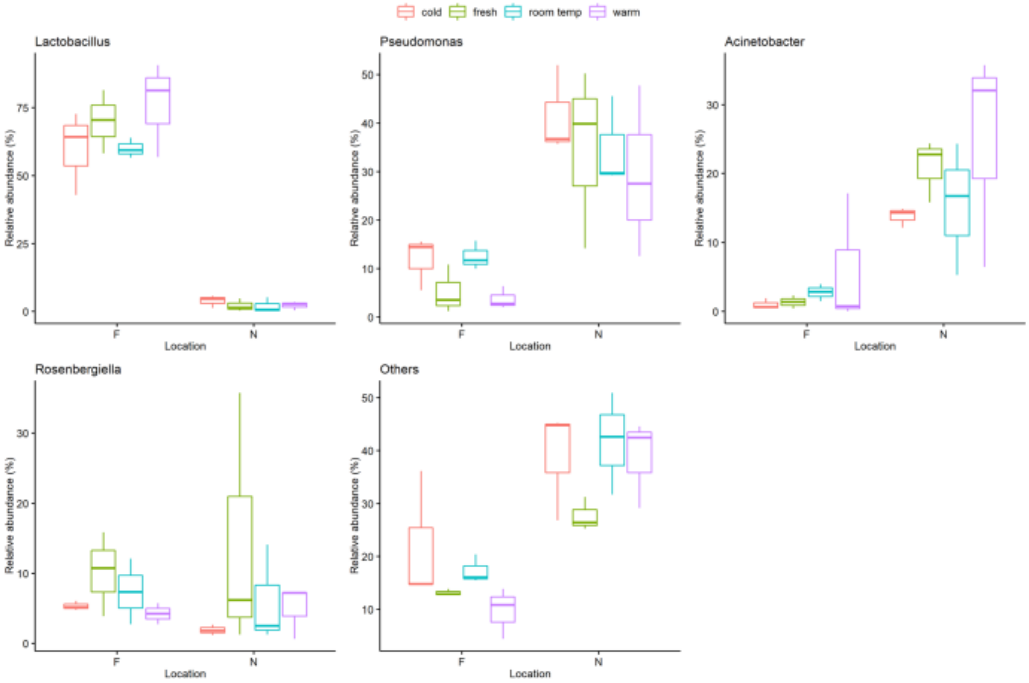

Fig. 2 Box plots chart (created with R 3.6.2), showing that the empirical distribution of bacterial genera differs between locations and storage conditions. The estimated interquartile range is represented as a box and a line spans from the observed minimum to the observed maximum

contrast, the source location of the pollen samples was associated with a significant difference in relative abundance of all bacterial genera (GLM; $p < 0.001$), except *Rosenbergiella* (GLM; $p = 0.264$) (Table S6). Location F was characterized by a high proportion of *Lactobacillus* (60–76%) and low proportion of *Pseudomonas* (5–12%), while location N showed low proportion of *Lactobacillus* (2–4%) and high proportion of *Pseudomonas* (29–42%) (Fig. 1a).

The fungal phyla from Dataset 2 are composed of 71% *Ascomycota*, 21% *Basidiomycota*, 0.15% *Motiellellomycota*, and unclassified (3.1%) (Table 4). The phylum *Ascomycota* consisted of 32 different fungal genera that were found in all samples. The main representatives of the *Ascomycota* genera in all analyzed samples were *Cladosporium* (10–45%), *Podosphaera* (1–30%), *Mycosphaerella* (5–20%), and *Zygosaccharomyces* (0–50%) (Fig. 1b) (Table S5). Significant differences between the storage conditions could be shown for fungal communities (GLM; $p < 0.001$), except for *Podosphaera* (GLM; $p = 0.084$) (Table S8). *Zygosaccharomyces* and *Aspergillus* showed significant differences between "fresh – warm," "cold – warm," and "room temperature – warm" conditions (Tukey; $p < 0.001$) (Table S9), while *Cladosporium* showed significant differences between "fresh – warm" and "room temperature – warm" conditions (Tukey; $p < 0.001$). The proportion of *Cladosporium* under "fresh" and "room temperature" storage conditions was significantly higher than under "warm" conditions (location N), while proportions of *Zygosaccharomyces* and *Aspergillus* significantly increased under warm storage conditions in all samples (Fig. 3). Furthermore, all locations showed also significant differences for every fungal genus (GLM; $p < 0.001$).

Discussion

The microorganism composition in bee pollen is affected by plant source, geographical origin, and bee keeper activities (Nogueira et al. 2012; De-Melo et al. 2016). In this study, we analyzed the botanical origin of the collected bee pollen samples. The pollen composition differed in all pollen samples; therefore, the influence of the botanical origin on the microorganism composition can be supported by the results of this study. We showed as well that the location has a significant influence on the bacterial and fungal communities associated with fresh, bee-collected pollen (GLM; $p < 0.001$). In addition, the composition and the changes of composition of microorganisms are influenced by the

Table 4 Fungal phyla composition with fungal genera calculated with total reads in all samples (Dataset 2)

Ascomycota (71%)	*Alpinaria*
	Alternaria
	Aspergillus
	Aureobasidium
	Bettsia
	Blumeria
	Cladosporium
	Debaryomyces
	Didymella
	Epicoccum
	Erysiphe
	Fusarium
	Geosmithia
	Leptosphaeria
	Metschnikowia
	Monilinia
	Monodictys
	Mycosphaerella
	Neodevriesia
	Neosetophoma
	Penicillium
	Periconia
	Phaeotheca
	Podosphaera
	Pseudoophiobolus
	Pyrenophora
	Ramularia
	Septoria
	Taphrina
	Tetracladium
	Trichomerium
	Zygosaccharomyces
Basidiomycota (21%)	
Motiellellomycota (0.15%)	
Unclassified (3.1%)	

geographical origin of the pollen samples. However, we only included two different location sites for analysis. In order to support this statement, future studies have to be done with a larger number of samples. Major changes of microorganism composition in bee pollen occurred during storage under simulated "warm" conditions. Therefore, this study confirms our hypothesis that different storage conditions have a significant effect on the composition of microorganisms in pollen. The results demonstrate clearly that pollen has to be removed from the trap and processed immediately to prevent unwanted microorganism growing, instead of leaving pollen in the trap during hot environmental conditions. The results of this study are based on the abundances of sequences; culture-based experiments should also be followed in further studies to support the statements of this study.

Interestingly, the effect of the storage conditions on the microbial communities seems to be different for bacteria and fungi. With regard to the bacterial composition, a significant difference between storage conditions could only be identified for *Lactobacillus* (GLM; $p = 0.039$). *Lactobacillus*, represented by the species *Lactobacillus kunkeii*, is a core gut bacterium that has been found in all of the analyzed samples and could be detected in earlier studies. It is also present in corbicula pollen, "beebread," and in floral nectar (Anderson et al. 2013; Kwong and Moran 2016). The proportion of *Lactobacillus* changes significantly between room temperature and warm conditions (Tukey; $p = 0.043$). Nevertheless, differences between storage conditions could also be determined for other bacteria. *Acinetobacter* is a bacterium needing aerobic growth condition and has been found not only in different environments, mainly in nectar of plants, but also in corbicula pollen, beebread, and in the intestinal tract of honey bees (*Apis mellifera*) (Fridman et al. 2012; Kim et al. 2014; Donkersley et al. 2018; Disayathanoowat et al. 2020). A small increase of *Acinetobacter* was observed during storage in warm conditions. Other studies also showed an increase of *Acinetobacter* in in-hive stored "bee bread" because it prefers a sugar-rich habitat (Fridman et al. 2012; Disayathanoowat et al. 2020). Two bacterial genera that are commonly found in plant material like nectar are *Pseudomonas* and *Rosenbergiella* (Fridman et al. 2012; Halpern et al. 2013). In contrast to fresh pollen, both tended to show a slight decrease during storage under warm conditions. Based on these results, it is reasonable to assume that beside changes in temperature, other factors that might influence the growth of bacteria have to be considered. Previous studies showed that the bacterial population tends to decrease under long-term storage in-hive, related to the low pH value in the hive (Anderson et al. 2014; Disayathanoowat et al. 2020). Environments with a low pH value show a high concentration of hydrogen ions, which tends to reduce bacterial growth, whereas the growth of fungi is increased (Rousk et al. 2009).

In contrast to the bacteria, we observed a consistently high influence of the storage conditions on the changes in fungal genus composition (GLM; $p < 0.001$). The fungus *Cladosporium* was the most abundant fungus in the freshly collected samples. It is ubiquitously found in indoor and outdoor environments such as air and soil (Zalar et al. 2007). In this study, we showed that the relative fraction of *Cladosporium* decreases during storage, especially under warm conditions. The fungal genera *Podosphaera* and *Mycosphaerella* can be isolated from plant environments and are both known to be plant pathogens (Crous et al. 2006; Baiswar et al. 2010; Garibaldi et al. 2012). Both were identified in freshly collected pollen as well as in stored pollen, but their proportion generally decreased during storage.

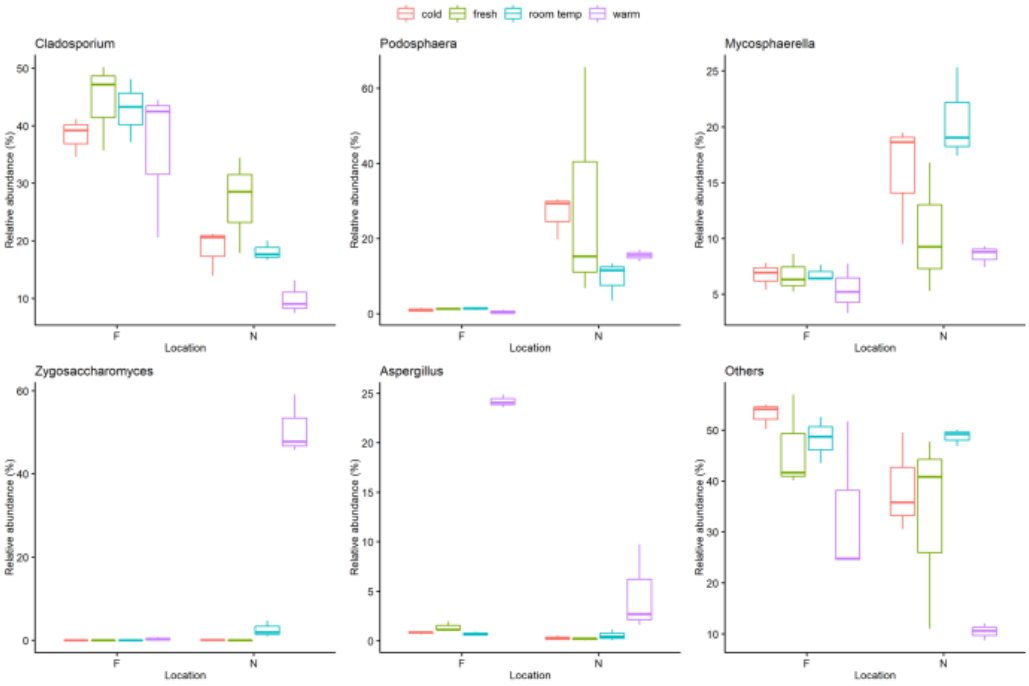

Fig. 3 Box plots chart (created with R 3.6.2), showing that the empirical distribution of fungal genera differs between locations and storage conditions. The estimated interquartile range is represented as a box and a line spans from the observed minimum to the observed maximum

However, several groups of fungi have been identified that grown more strongly under warm and humid storage conditions, especially *Zygosaccharomyces* and *Aspergillus*. Previous studies have also shown that fungal composition changes during storage of bee bread (Sinpoo et al. 2017; Detry et al. 2020; Disayathanoowat et al. 2020). Sinpoo et al. (2017) demonstrated that the high diversity of fungal communities in bee bread decreased significantly during storage time. The most dominated fungal species in corbicular pollen were *Cladosporium* and *Aspergillus*, whereas also *Zygosaccharomyces* dominated in stored bee bread. Detry et al. (2020) also showed that the high abundance of yeast decreased within increasing storage time. However, also the yeast *Zygosaccharomyces* dominated clearly in aged bee bread. The yeast genus *Zygosaccharomyces* is very osmotolerant yeast species and has a high tolerance for different sugars. Therefore, it is known as notorious spoilage organism of sugar-rich foods and beverages such as candy, fruit juices, sugar syrups, and wine (Martorell et al. 2007; Zuehlke et al. 2013; Aneja et al. 2014; Marvig et al. 2014). *Zygosaccharomyces spp.* are also normal members of the fungal gut communities of honey bees (Yun et al. 2018); consequently, they can be transmitted from the bee saliva to the pollen. In this study, *Zygosaccharomyces spp.* was identified as the most prolifically growing microorganisms in bee pollen stored under warm temperatures. Therefore, it seems likely that *Zygosaccharomyces* can spoil bee pollen in warm and humid storage conditions, as they producing ethanol or carbon dioxide from sugar. Pollen, used as food supplement for human, should not contain any spoilage yeast, otherwise the aroma and sensory can be influenced by fermentation (Sperber and Doyle 2009). However, since the yeast is probably already being transferred from the bees to the pollen, it is very important to prevent their reproduction and growth. The growth of such yeasts can be prevented by freezing or cooling. In our study, we did not observe any increase in the *Zygosaccharomyces* proportion at 4 °C. Other studies showed as well that *Zygosaccharomyces* growth is clearly reduced even at 8 °C (Marvig et al. 2014). Also, an increase in the *Aspergillus* proportion in the samples stored under warm conditions has to be observed with regard to human nutrition. *Aspergillus* is extremely halo- and osmotolerant (Stratford et al. 2019) and the genus contains a number of highly mycotoxigenic species (González et al. 2005). Some species of this fungal genus have been identified as pathogens in insects, animals, and humans (Foley et al. 2014; Dagenais and

Keller 2009). In food, *Aspergillus spp.* can spoil as visible growth of black mold, discoloration, or in producing mycotoxins. The effects of mycotoxins on human health are complex and can cause cancerogenic effects or central nervous system damage (Sperber and Doyle 2009). Since the growth of *Aspergillus* can cause major effects on human health, it is particularly important to prevent the growing in pollen samples. Another study reported findings of mycotoxin-producing *Aspergillus spp.* in "ready-to-eat" pollen samples from Spain (González et al. 2005). The fungal contamination in the study from González et al. indicated that the post-harvest pollen processed negatively impacted pollen quality. Incorrect storage or drying conditions as well as non-daily harvest were pointed out as reasons for the contamination.

In conclusion, pollen stored under warm conditions showed the clearest changes in fungal composition, compared to the freshly collected pollen (Tukey; $p < 0.001$). Growth of fungi from the genera *Zygosaccharomyces* or *Aspergillus* was likely the cause of spoilage. Therefore, during processing of freshly harvested bee pollen, it is important to prevent growth of these spoilage microorganisms. This is most conveniently achieved by harvesting daily, followed by processing the pollen directly to refrigeration or, even better, freezing.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-021-13932-4.

Acknowledgements The authors thank Dr. Raghdan Alkattea for help with the palynological analysis. Furthermore, the authors thank the volunteer beekeepers for sample collection.

Data Availability of data and materials The authors confirm the data generated or analyzed during this study are included in this published article and its supplementary information files. All datasets (including the raw data and sequences) used and analyzed during this current study are also available from the corresponding author on reasonable request.

Author contribution Carolin Friedle: conceptualization, methodology, data curation, formal analysis, resources, visualization, writing — original draft, and writing — review & editing; Paul D'Alvise: methodology, data curation, formal analysis, and writing — review & editing; Karsten Schweikert: data curation, formal analysis, visualization, and writing — review & editing; Klaus Wallner: conceptualization, data curation, funding acquisition, supervision; writing — review & editing; Martin Hasselmann: conceptualization, methodology, data curation, supervision, and writing — review & editing.

Funding Open Access funding enabled and organized by Projekt DEAL. This study was supported by the Ministry of Rural Areas and Consumer Protection Baden-Wuerttemberg.

Declarations

Ethics approval The authors confirm that ethical standards were addressed.

Consent to participate The authors confirm the volunteer's declaration of consent.

Consent to publish The authors confirm the volunteer's consent for publication. The map generated by the Environmental Information System (UIS) of the LUBW State Institute for the Environment Baden-Wuerttemberg and the State Office for Geoinformation and Rural Development Baden-Wuerttemberg have been given permission to be published by mentioning them as basic data.

Competing interests The authors declare no competing interests.

References

Anderson KE, Sheehan TH, Mott BM, Maes P, Snyder L, Schwan MR, Walton A, Jones BM, Corby-Harris V (2013) Microbial ecology of the hive and pollination landscape: bacterial associates from floral nectar, the alimentary tract and stored food of honey bees (Apis mellifera). PLoS One 8(12):e83125. https://doi.org/10.1371/journal.pone.0083125

Anderson KE, Carroll MJ, Sheehan T, Lanan MC, Mott BM, Maes P, Corby-Harris V (2014) Hive-stored pollen of honey bees: many lines of evidence are consistent with pollen preservation, not nutrient conversion. Mol Ecol 23(23):5904–5917. https://doi.org/10.1111/mec.12966

Aneja KR, Dhiman R, Aggarwal NK, Aneja A (2014) Emerging preservation techniques for controlling spoilage and pathogenic microorganisms in fruit juices. Int J Microbiol 2014(7):1–14. https://doi.org/10.1155/2014/758942

Avni D, Hendriksma HP, Dag A, Uni Z, Shafir S (2014) Nutritional aspects of honey bee-collected pollen and constraints on colony development in the eastern Mediterranean. J Insect Physiol 69:65–73. https://doi.org/10.1016/j.jinsphys.2014.07.001

Baiswar P, Chandra S, Kumar R, Ngachan SV, Munda GC (2010) First report of powdery mildew caused by Podosphaera sp. on Hibiscus sabdariffa in India. Austral Plant Dis Notes 5(1):123. https://doi.org/10.1071/DN10045

Bárbara M, Machado C, Sodré G, Dias L, Estevinho L, de Carvalho C (2015) Microbiological assessment, nutritional characterization and phenolic compounds of bee pollen from Mellipona mandacaia Smith, 1983. Molecules 20(7):12525–12544. https://doi.org/10.3390/molecules200712525

Bonvehí JS, Jordà RE (1997) Nutrient composition and microbiological quality of honeybee-collected pollen in Spain. J Agric Food Chem (45):725–732. https://doi.org/10.1021/jf960265q

Carpes ST, Mourao GB, Alencar SM, Masson ML (2009) Chemical composition and free radical scavenging activity of Apis mellifera bee pollen from Southern Brazil. Braz J Food Technol (03):220–229. https://doi.org/10.4260/BJFT2009800900016

Corby-Harris V, Maes P, Anderson KE (2014) The bacterial communities associated with honey bee (Apis mellifera) foragers. PLoS One 9(4): e95056. https://doi.org/10.1371/journal.pone.0095056

Cribari-Neto F, Zeileis A (2010) Beta regression in R. J Stat Softw (43). https://doi.org/10.18637/jss.v034.i02

Cridge AG, Leask MP, Duncan EJ, Dearden PK (2015) What do studies of insect polyphenisms tell us about nutritionally-triggered epigenomic changes and their consequences? Nutrients 7(3):1787–1797. https://doi.org/10.3390/nu7031787

Crous PW, Wingfield MJ, Mansilla JP, Alfenas AC, Groenewald JZ (2006) Phylogenetic reassessment of Mycosphaerella spp. and their anamorphs occurring on Eucalyptus. II. Stud Mycol 55:99–131. https://doi.org/10.3114/sim.55.1.99

D'Alvise P, Böhme F, Codrea MC, Seitz A, Nahnsen S, Binzer M, Rosenkranz P, Hasselmann M (2018) The impact of winter feed type on intestinal microbiota and parasites in honey bees. Apidologie 49(2):252–264. https://doi.org/10.1007/s13592-017-0551-1

Dagenais TRT, Keller NP (2009) Pathogenesis of Aspergillus fumigatus in Invasive Aspergillosis. CMR 22(3):447–465. https://doi.org/10.1128/CMR.00055-08

De-Melo AAM, Estevinho MLMF, Almeida-Muradian LB (2015) A diagnosis of the microbiological quality of dehydrated bee-pollen produced in Brazil. Lett Appl Microbiol 61(5):477–483. https://doi.org/10.1111/lam.12480

De-Melo AAM, Estevinho MLMF, Sattler JAG, Souza BR, Freitas AS, Barth OM, Almeida-Muradian LB (2016) Effect of processing conditions on characteristics of dehydrated bee-pollen and correlation between quality parameters. LWT Food Sci Technol 65:808–815. https://doi.org/10.1016/j.lwt.2015.09.014

Detroy BF, Harp ER (1976) Pollen traps: trapping pollen from honey bee colonies. Agricultural Research Service. United States Department ofAgricultural. Prod Res Rep Madison (163)

Detry R, Simon-Delso N, Bruneau E, Daniel H-M (2020) Specialisation of yeast genera in different phases of bee bread maturation. Microorganisms 8(11). https://doi.org/10.3390/microorganisms8111789

Dinkov D (2016) Microorganisms in vacuum stored flower bee pollen. J Bacteriol Virol 46(4):258. https://doi.org/10.4167/jbv.2016.46.4.258

Dinkov D (2018) Isolation and identification of bacteria in flower bee pollen. Walailak J Sci Tech 15(3):225–234

Disayathanoowat T, Li H, Supapimon N, Suwannarach N, Lumyong S, Chantawannakul P, Guo J (2020) Different dynamics of bacterial and fungal communities in hive-stored bee bread and their possible roles: a case study from two commercial honey bees in China. Microorganisms 8(2). https://doi.org/10.3390/microorganisms8020264

Donkersley P, Rhodes G, Pickup RW, Jones KC, Wilson K (2018) Bacterial communities associated with honeybee food stores are correlated with land use. Ecol Evol 8(10):4743–4756. https://doi.org/10.1002/ece3.3999

Edgar RC (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods 10(10):996–998. https://doi.org/10.1038/nmeth.2604

Estevinho LM, Rodrigues S, Pereira AP, Feás X (2012) Portuguese bee pollen: palynological study, nutritional and microbiological evaluation. Int J Food Sci Technol 47(2):429–435. https://doi.org/10.1111/j.1365-2621.2011.02859.x

Fatrcová-Šramková K, Nôžková J, Máriássyová M, Kačániová M (2016) Biologically active antimicrobial and antioxidant substances in the Helianthus annuus L. bee pollen. J Environ Sci Health B 51(3):176–181. https://doi.org/10.1080/03601234.2015.1108811

Feás X, Vázquez-Tato MP, Estevinho L, Seijas JA, Iglesias A (2012) Organic bee pollen: botanical origin, nutritional value, bioactive compounds, antioxidant activity and microbiological quality. Molecules 17(7):8359–8377. https://doi.org/10.3390/molecules17078359

Ferrari S, Cribari-Neto F (2004) Beta regression for modelling rates and proportions. J Appl Stat 31(7):799–815. https://doi.org/10.1080/0266476042000214501

Foley K, Fazio G, Jensen AB, Hughes WOH (2014) The distribution of Aspergillus spp. opportunistic parasites in hives and their pathogenicity to honey bees. Vet Microbiol 169(3-4):203–210. https://doi.org/10.1016/j.vetmic.2013.11.029

Fridman S, Izhaki I, Gerchman Y, Halpern M (2012) Bacterial communities in floral nectar. Environ Microbiol Rep 4(1):97–104. https://doi.org/10.1111/j.1758-2229.2011.00309.x

Friedle C, Wallner K, Rosenkranz P, Martens D, Vetter W (2021) Pesticide residues in daily bee pollen samples (April–July) from an intensive agricultural region in Southern Germany. Environ Sci Pollut Res:1–15. https://doi.org/10.1007/s11356-020-12318-2

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. Mol Ecol 2(2):113–118. https://doi.org/10.1111/j.1365-294x.1993.tb00005.x

Garibaldi A, Bertetti D, Pensa P, Poli A, Gullino ML (2012) Podosphaera sp. on Euphorbia susannae and E. inermis in Italy. Plant Dis 96(12): 1824. https://doi.org/10.1094/PDIS-06-12-0569-PDN

González G, Hinojo MJ, Mateo R, Medina A, Jiménez M (2005) Occurrence of mycotoxin producing fungi in bee pollen. Int J Food Microbiol 105(1):1–9. https://doi.org/10.1016/j.ijfoodmicro.2005.05.001

Halpern M, Fridman S, Atamna-Ismaeel N, Izhaki I (2013) Rosenbergiella nectarea gen. nov., sp. nov., in the family Enterobacteriaceae, isolated from floral nectar. Int J Syst Evol Microbiol 63(Pt 11):4259–4265. https://doi.org/10.1099/ijs.0.052217-0

Hani B, Dalila B, Saliha D, Daoud H, Mouloud G, Seddik K (2012) Microbioligical sanitary aspects of pollen. Adv Environ Biol (6): 1415–1420. ISSN 1995-0756

Kevan PG, Baker HG (1983) Insects as flower visitors and pollinators. Annu Rev Entomol 28(1):407–453. https://doi.org/10.1146/annurev.en.28.010183.002203

Kim PS, Shin N-R, Kim JY, Yun J-H, Hyun D-W, Bae J-W (2014) Acinetobacter apis sp. nov., isolated from the intestinal tract of a honey bee, Apis mellifera. J Microbiol 52(8):639–645. https://doi.org/10.1007/s12275-014-4078-0

Kisand V, Wikner J (2003) Limited resolution of 16S rDNA DGGE caused by melting properties and closely related DNA sequences. J Microbiol Methods 54(2):183–191. https://doi.org/10.1016/S0167-7012(03)00038-1

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 41(1):e1. https://doi.org/10.1093/nar/gks808

Kostić AŽ, Barać MB, Stanojević SP, Milojković-Opsenica DM, Tešić ŽL, Šikoparija B, Radišić P, Prentović M, Pešić MB (2015) Physicochemical composition and techno-functional properties of bee pollen collected in Serbia. LWT Food Sci Technol 62(1):301–309. https://doi.org/10.1016/j.lwt.2015.01.031

Kwong WK, Moran NA (2016) Gut microbial communities of social bees. Nature reviews. Microbiology 14(6):374–384. https://doi.org/10.1038/nrmicro.2016.43

Lagkouvardos I, Joseph D, Kapfhammer M, Giritli S, Horn M, Haller D, Clavel T (2016) IMNGS: a comprehensive open resource of processed 16S rRNA microbial profiles for ecology and diversity studies. Sci Rep 6:33721. https://doi.org/10.1038/srep33721

Lindauer M (1952) A contribution to the question of the division of labor in the bee state. (Ein Beitrag zur Frage der Arbeitsteilung im

Bienenstaat). Z Vergl Physiol 34(4):299–345. https://doi.org/10.1007/BF00298048

Manirajan BA, Ratering S, Cardinale M, Maisinger C, Schnell S (2019) The microbiome of flower pollen and its potential impact on pollen-related allergies. The flowering of plant microbiome and the human connection. Institute of Applied Microbiology, Justus-Liebig University, Giessen, Germany

Martins MCT, Morgano MA, Vicente E, Baggio SR, Rodriguez-Amaya DB (2011) Physicochemical composition of bee pollen from eleven Brazilian States. J Agric Sci (55):107–116. ISSN 1643-4439

Martorell P, Stratford M, Steels H, Fernández-Espinar MT, Querol A (2007) Physiological characterization of spoilage strains of Zygosaccharomyces bailii and Zygosaccharomyces rouxii isolated from high sugar environments. Int J Food Microbiol 114(2):234–242. https://doi.org/10.1016/j.ijfoodmicro.2006.09.014

Marvig CL, Kristiansen RM, Madsen MG, Nielsen DS (2014) Identification and characterisation of organisms associated with chocolate pralines and sugar syrups used for their production. Int J Food Microbiol 185:167–176. https://doi.org/10.1016/j.ijfoodmicro.2014.05.017

Mauriello G, de Prisco A, Di Prisco G, La Storia A, Caprio E (2017) Microbial characterization of bee pollen from the Vesuvius area collected by using three different traps. PLoS One 12(9):e0183208. https://doi.org/10.1371/journal.pone.0183208

Moreno Andrade VD, Saldaña Gutiérrez C, Calvillo Medina RP, Cruz Hérnandez A, Vázquez Cruz MA, Torres Ruíz A, Romero Gómez S, Ramos López MA, Álvarez-Hidalgo E, López-Gaytan SB, Ramírez NS, Jones GH, Hernandez-Flores JL, Campos-Guillén J (2018) Microbial diversity in commercial bee pollen from Europe, Chile, and Mexico, based on 16S rRNA gene amplicon metagenome sequencing. Genome Announc 6(20). https://doi.org/10.1128/genomeA.00247-18

Nicolson SW, Human H (2013) Chemical composition of the 'low quality' pollen of sunflower (Helianthus annuus, Asteraceae). Apidologie 44(2):144–152. https://doi.org/10.1007/s13592-012-0166-5

Nogueira C, Iglesias A, Feás X, Estevinho LM (2012) Commercial bee pollen with different geographical origins: a comprehensive approach. Int J Mol Sci 13(9):11173–11187. https://doi.org/10.3390/ijms130911173

Paramás AMG, Bárez JAG, Marcos CC, García-Villanova RJ, Sánchez JS (2005) HPLC-fluorimetric method for analysis of amino acids in products of the hive (honey and bee-pollen). Food Chem 95(1):148–156. https://doi.org/10.1016/j.foodchem.2005.02.008

Pascoal A, Rodrigues S, Teixeira A, Feás X, Estevinho LM (2014) Biological activities of commercial bee pollens: antimicrobial, antimutagenic, antioxidant and anti-inflammatory. Food Chem Toxicol 63:233–239. https://doi.org/10.1016/j.fct.2013.11.010

Rousk J, Brookes PC, Bååth E (2009) Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. Appl Environ Microbiol 75(6):1589–1596. https://doi.org/10.1128/AEM.02775-08

Seeburger VC, D'Alvise P, Shaaban B, Schweikert K, Lohaus G, Schroeder A, Hasselmann M (2020) The trisaccharide melezitose impacts honey bees and their intestinal microbiota. PLoS One 15(4):e0230871. https://doi.org/10.1371/journal.pone.0230871

Sinpoo C, Williams GR, Chantawannakul P (2017) Dynamicy of fungal communities in corbicular pollen and bee bread. Chiang Mai J Sci 44(4):1235–1247

Sperber WH, Doyle MP (2009) Compendium of the microbiological spoilage of foods and beverages. Springer New York, New York

Stratford M, Steels H, Novodvorska M, Archer DB, Avery SV (2019) Extreme osmotolerance and halotolerance in food-relevant yeasts and the role of glycerol-dependent cell individuality. Front Microbiol 9:2179. https://doi.org/10.3389/fmicb.2018.03238

Taha E-KA, Al-Kahtani S, Taha R (2019) Protein content and amino acids composition of bee-pollens from major floral sources in Al-Ahsa, eastern Saudi Arabia. Saudi J Biol Sci 26(2):232–237. https://doi.org/10.1016/j.sjbs.2017.06.003

Turner S, Pryer KM, Miao Vivian PW, Palmer JD (1999) Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. J Eukaryot Microbiol 46:327–338

White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. Evolution PCR Protocols: A Guide to Methods and Applications:315–322

Willmer P (2011) Pollination and floral ecology. Princeton University Press, New Jersey

Yun J-H, Jung M-J, Kim PS, Bae J-W (2018) Social status shapes the bacterial and fungal gut communities of the honey bee. Sci Rep 8(1):2019. https://doi.org/10.1038/s41598-018-19860-7

Zalar P, de Hoog GS, Schroers H-J, Crous PW, Groenewald JZ, Gunde-Cimerman N (2007) Phylogeny and ecology of the ubiquitous saprobe Cladosporium sphaerospermum, with descriptions of seven new species from hypersaline environments. Stud Mycol 58:157–183. https://doi.org/10.3114/sim.2007.58.06

Zuehlke JM, Petrova B, Edwards CG (2013) Advances in the control of wine spoilage by Zygosaccharomyces and Dekkera/Brettanomyces. Annu Rev Food Sci Technol 4(1):57–78. https://doi.org/10.1146/ANNUREV-FOOD-030212-182533

3.3 High abundance of pyrrolizidine alkaloids in bee pollen collected in July 2019 from Southern Germany

Publiziert in: *Environmental Monitoring and Assessment*

Abstract

Pyrrolizidine alkaloids (PA) are secondary plant defense compounds and known pre-toxins when containing a 1,2-double bound. They are commonly produced by various plants and may thus be present in bee pollen which may be consumed by humans as food supplements. In this study, PA were determined in bee pollen samples from 57 locations in Southern Germany sampled by means of pollen traps in July 2019. Samples were analyzed by using palynological methodology and solid phase (SPE)-extraction followed by LC-MS/MS. In total, 52 pollen samples featured total pyrrolizidine alkaloids (ΣPA) with concentrations up to 48,000 ng/g bee pollen, while the N-oxides (NO) echinatine-NO and rinderine-NO clearly dominated. In contrast, the palynological analysis only detected 33 samples with pollen from PA producing plants. Accordingly, the results showed that palynological analysis is not sufficient to determine PA in pollen. In addition, a risk assessment was followed to estimate the risk of the detected PA concentrations to humans.

Friedle, C., Kapp, T., Wallner, K., Alkattea, R., Vetter, W. (2022): High abundance of pyrrolizidine alkaloids in bee pollen collected in July 2019 from Southern Germany. In: *Environ Moni and Ass.* DOI: 10.1007/s10661-022-09907-8.

Environ Monit Assess (2022) 194:250
https://doi.org/10.1007/s10661-022-09907-8

High abundance of pyrrolizidine alkaloids in bee pollen collected in July 2019 from Southern Germany

Carolin Friedle · Thomas Kapp · Klaus Wallner · Raghdan Alkattea · Walter Vetter

Received: 18 June 2021 / Accepted: 25 February 2022

Abstract Pyrrolizidine alkaloids (PA) are secondary plant defense compounds and known pre-toxins when containing a 1,2-double bond. They are commonly produced by various plants and may thus be present in bee pollen which may be consumed by humans as food supplements. In this study, PA were determined in bee pollen samples from 57 locations in Southern Germany sampled by means of pollen traps in July 2019. Samples were analyzed by using palynological methodology and solid-phase extraction (SPE) followed by LC–MS/MS. In total, 52 pollen samples featured total pyrrolizidine alkaloids (ΣPA) with concentrations up to 48,000 ng/g bee pollen, while the N-oxides (NO) echinatine-NO and rinderine-NO clearly dominated. In contrast, the palynological analysis only detected 33 samples with pollen from PA-producing plants. Accordingly, the results showed that palynological analysis is not sufficient to determine PA in pollen. In addition, a risk assessment was followed to estimate the risk of the detected PA concentrations to humans.

Keywords Pyrrolizidine alkaloids · Bee-collected pollen · Palynological analysis · LC–MS/MS · Risk management

Introduction

From early spring to late summer, honey bees (*Apis mellifera*) are collecting pollen from various plants. Being rich in protein, fatty acids, and vitamins, bee pollen is the primary nutritional source for bees (Avni et al., 2014; Mărgăoan et al., 2014; Taha et al., 2019). Due to these valuable ingredients, bee pollen is also an attractive food supplement in human nutrition (Feás et al., 2012). For this purpose, bee pollen can be collected by the installation of pollen traps at the hive from early spring (usually April to June) to late summer. However, a thorough control of samples is important because bee pollen may be contaminated with both residues of pesticides applied in agriculture mainly during spring (April to June) (Böhme et al., 2018; Drummond et al., 2018; Friedle et al., 2021; Traynor et al., 2016) and also harmful natural contaminants produced by plants during summer (June to August) (BfR, 2013).

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s10661-022-09907-8.

C. Friedle (✉) · K. Wallner · R. Alkattea
Apicultural State Institute, University of Hohenheim, Stuttgart, Germany
e-mail: carolin_friedle@uni-hohenheim.de

T. Kapp
Chemical and Veterinary Analysis Agency (CVUA), Stuttgart, Fellbach, Germany

W. Vetter
Institute of Food Chemistry (170B), University of Hohenheim, Stuttgart, Germany

Published online: 06 March 2022

One class of potential natural contaminants of bee pollen is pyrrolizidine alkaloids (PA) (EFSA, 2011, 2017). PA are a group of secondary plant defense compounds whose common structural element is a 1-azabicyclo[3.3.0]octane (pyrrolizidine) backbone which usually carries an additional 1,2-double bond (present in all toxic variants) along with a hydroxymethyl substituent in the 1-position and a hydroxyl group in the 7-position, respectively (Fig. 1a). The resulting PA core is the so-called necine base which is either esterified once (monoesters) or twice (diesters or cyclic diesters) with acyl moieties varying in structure and stereochemistry (Fig. 1b). Likewise, the stereocenter in 7-position (*) can be both *R*- or *S*-configurated (Fig. 1a). Last but not least, virtually all PA exist in two distinct forms, e.g., tertiary heterocyclic amines and the corresponding N-oxide form (PANO) (Fig. 1c). These structural variations give rise to more than 600 structurally different PA (EFSA, 2011). In the following, the designation "PA" is also used as summarizing term for PA and PANO. Assumedly, around 3% of all flowering plants may produce PA (Smith & Culvenor, 1981). Most of these plants belong to the families of *Asteraceae* (e.g., *Senecioneae* (*Senecio* spp.) and *Eupatorieae* (*Eupatorium* spp.)), *Boraginaceae* (e.g., *Borago* spp. and *Echium* spp.), and *Fabaceae* (e.g., *Crotalaria* spp.) (EFSA, 2011; Hartmann & Toppel, 1987).

Harmful PA are hepatotoxic to both animals and humans and can cause acute poisoning and chronic effects (Colegate et al., 2012; EFSA, 2011; Kakar et al., 2010). Chronic exposure to low PA amounts

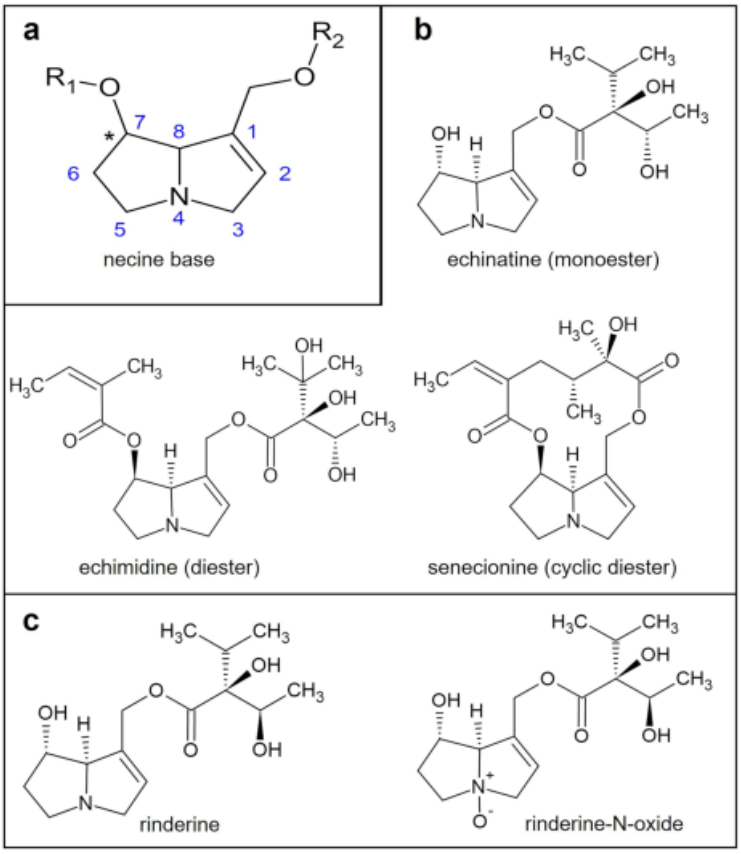

Fig. 1 **a** Structures of necine base. **b** Examples of PA with different kinds of esterification. **c** A PA structure (rinderine) with the corresponding N-oxide (PANO)

can result in diseases such as liver cirrhosis or possibly cause cancer as metabolic activation produces genotoxic and cancerogenic metabolites (Edgar et al., 2011). As a consequence, the US Food and Drug Administration (FDA) decreed to ban PA-containing products from the market (Food & Drug Administration, 2001). By contrast, maximum levels of PA are currently not in force for food in the European Union (EU). A novel EU regulation defining maximum PA levels for pollen and pollen-based food supplements among other foods such as (herbal) teas, herbs, and spices will be effective by July 2022. Then, for pollen and related food supplements a maximum PA level of 500 µg/kg must not be exceeded (European Commission, 2020). Furthermore, a maximum intake of 1 µg PA per day was established in pharmaceutical products (Federal Institute for Pharmaceuticals and Medical Products, 2016). Likewise, the European Food Safety Authority (EFSA) has introduced a benchmark dose lower confidence limit 10% ($BMDL_{10}$) amount of 237 µg riddelliine per kg body weight (BW) in rats as a reference point for the assessment of carcinogenic risks, assuming similar carcinogenic potency for different PA (EFSA, 2017). Considering a tolerable margin of exposure (MOE) of 10,000 for humans, this corresponds with a maximum intake of 0.024 µg PA per kg BW per day (BfR, 2020). This in turn corresponds with a maximum daily intake of 1.8 µg PA per day for adults (75 kg BW) and ~1 µg for juveniles (40 kg BW, 10 years) (GBE, 2017).

Next to foods with a direct botanical background such as teas and herbals, PA were also exemplarily studied in honey and bee pollen (Bodi et al., 2014; Boppré et al., 2005; Dübecke et al., 2011; Gottschalk et al., 2018; Kaltner et al., 2020; Kast et al., 2018, 2019; Kempf et al., 2010, 2011; Martinello et al., 2014; Orantes-Bermejo et al., 2013; Roeder, 1995). In one study, 17 of 55 commercial pollen products, mainly from Spain, Romania, Italy, and France, were contaminated with toxic PA of up to 16,400 ng/g pollen (Kempf et al., 2010). Similarly, Dübecke et al. (2011) detected PA in 60% of 119 bee pollen samples from various countries with ΣPA contents of up to 37,900 ng/g pollen. Recently, Kast et al. (2018) presented two complementary methods: a high-performance liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) method which allowed studying 18 PA and PANO in commercial bee pollen products as well as daily collected bee pollen samples, and a LC-HRMS method comparing daily collected pollen samples to flower heads, by detecting of all PA types, including saturated, non-cancerogenic PA. To achieve higher confidence for identification of the plant source, nearly all PA-containing plants occurring in Switzerland were also analyzed by LC-HRMS. Bee-collected pollen indicated the presence of highest PA concentrations in "*Echium*-type PA" samples collected in June and "*Eupatorium*-type PA" samples (assigned as intermedine and lycopsamine (-N-oxides)) mainly from mid-July and August (Kast et al., 2018). However, no reliable information existed about PA levels in bee-collected pollen from Germany.

Given the geographic neighborhood of Switzerland (the observation site Basel of Kast et al. (2018) is directly at the border to Germany) and Southern Germany, we aimed to carry out a thorough study by collecting bee pollen by means of traps in 57 locations in Baden-Wuerttemberg (Southern Germany). Knowing the fact that pollen samples in spring (from April to June) could be highly contaminated with pesticides in Southern Germany (Friedle et al., 2021), the aim was to determine whether the pollen samples were otherwise contaminated in a later period. In the end of July 2019 (July 15–August 1), bee pollen was daily collected on each site on 14 consecutive days, and then pooled. The pooled site samples were analyzed palynologically using a microscope to get insights into the botanical background of the samples. Chemical analysis was carried out with an optimized LC–MS/MS method which allowed determining 42 PA simultaneously. The results were used to evaluate whether bee pollen from Baden-Wuerttemberg (Southern Germany) can be safely consumed.

Materials and methods

Chemical reagents

PA standards used for analysis were 7-acetylintermedine, 7-acetylintermedine-N-oxide, 7-acetyllycopsamine, 7-acetyllycopsamine-N-oxide, echinatine, echinatine-N-oxide, heliosupine, heliosupine-N-oxide, heliotrine, heliotrine-N-oxide, indicine hydrochloride, indicine-N-oxide, integerrimine, integerrimine-N-oxide, intermedine, lycopsamine, lycopsamine-N-oxide, retrorsine, riddelliine, riddelliine-N-oxide, rinderine, rinderine-N-oxide,

senecionine, senecionine-N-oxide, seneciphylline, and senkirkine, from PhytoPlan (Heidelberg, Germany). Echimidine, echimidine-N-oxide, erucifoline, erucifoline-N-oxide, europine hydrochloride, europine-N-oxide, intermedine-N-oxide, jacobine, jacobine-N-oxide, lasiocarpine, monocrotaline-N-oxide, retrorsine-N-oxide, seneciphylline-N-oxide, senecivernine, and senecivernine-N-oxide were from PhytoLab (Vestenbergsgreuth, Germany). Lasiocarpine-N-oxide was from Cfm Oskar Tropitzsch (Marktredwitz, Germany). Monocrotaline was from Carl Roth (Karlsruhe, Germany) and trichodesmine from Latoxan (Valence, France). For the palynological analysis and sample preparation, the following reagents were used: demineralized water, ultrapure water, dish soap, Kaiser's glycerol gelatin, dry ice, 0.05 M sulfuric acid, ammonia solution, and methanol. For sample analysis, 5 mM ammonium formate and 0.1% (v/v) formic acid in methanol were used.

Sample collection

Bee pollen traps were installed on apiaries at 57 locations in Baden-Wuerttemberg, Southern Germany, to collect pollen loads from returning honey bees (*Apis mellifera*) (Fig. S1). All traps were installed on privately owned bee colonies of voluntary beekeepers, so no exact coordinates will be given and no permits were needed for this study. The collecting time (July 15–August 1) was adapted to the flowering phase of *Borago* sp., *Echium* sp., *Eupatorium* sp., and *Senecio* sp. at the end of July 2019. Pollen samples were collected daily within 14 consecutive days (rainy days uncounted) and mixed to one pooled sample per site. An aliquot of 45 g pooled pollen per site was taken and stored at −20 °C until preparation.

Sample preparation

Bee pollen samples were prepared following a validated solid-phase extraction (SPE) protocol originally developed by the BfR (2014). In brief, samples were brought up to room temperature and homogenized with dry ice in a mill (Retsch, Haan, Germany). An aliquot of 2.0 g ± 0.1 g bee pollen was weighted into a tube (Sarstedt, Nümbrecht, Germany), mixed with 20 mL 0.05 M sulfuric acid and extracted for 15 min in an ultrasonic bath. The samples were centrifuged (5 min, 3000 × *g*) and the supernatant was transferred to another tube. The pellet was used for a second extraction using 20 mL 0.05 M sulfuric acid and was re-extracted for 15 min in an ultrasonic bath. After centrifugation (5 min, 3000 × *g*) the supernatant was transferred to the first extract and adjusted to pH 6–7 with an ammonia solution. SPE was carried out with DSC-C18 SPE cartridges (500 mg, 6 mL, Supelco Merck, Darmstadt, Germany) in a vacuum chamber as follows. Conditioning was performed using 5 mL methanol, followed by 5 mL ultrapure water. The sample extract was loaded onto the cartridge (2 × 5 mL), followed by washing with 2 × 5 mL ultrapure water and 5 min drying under vacuum conditions. The sample was eluted with 5 mL methanol and evaporated to 1 mL in a heating block maintained at 55 °C under nitrogen flow. After addition of ultrapure water to a total volume of 10 mL, an aliquot was filled into an LC vial for analysis.

LC–MS/MS analysis of pyrrolizidine alkaloids

PA single component analysis on 42 PA/PANO was performed with a 6490 tandem mass spectrometer (Agilent Technologies, Waldbronn, Germany) coupled to a 1290 ultra-high-performance liquid chromatography (UHPLC) system (Agilent Technologies, Waldbronn, Germany) at the Chemical and Veterinary Analysis Agency (CVUA Stuttgart, Germany). Chromatographic separation was carried out on a 150 mm × 2.1 mm i.d., 1.7 µm particle-sized Acquity CSH C18 UPLC column (Waters, Eschborn, Germany), using aqueous 5 mM ammonium formate solution with 0.1% (v/v) formic acid as eluent A and 5 mM formate solution with 0.1% (v/v) formic acid in methanol as eluent B. Gradient elution started at 2.5% eluent B (1.0 min), linearly increased to 10% eluent B at 15.5 min, and then increased further to 23% eluent B at 20.5 min and in the next step to 36% eluent B at 25.0 min before being ramped to 100% B at 28.0 min (1.5 min). At 29.6 min, initial conditions were restored and kept until the final run time of 31.5 min. Flow rate was set at 0.35 mL min^{-1} at a controlled column temperature of 50 °C. The injection volume was 2 µL for all runs.

The Jet Stream electrospray ion source was operated in positive mode at a capillary voltage of 3500 V. The nebulizer pressure was set to 25 psi with a gas flow of 13 L min^{-1} at a temperature of 250 °C. Sheath gas flow rate was set to 12 L min^{-1} at a temperature of

360 °C. High- and low-pressure ion funnel RFs were set to 150 V and 60 V, respectively. For each analyte, three transitions were recorded in dynamic multiple reaction monitoring (dMRM) mode (Table 1). In dMRM mode, an acquisition window of 6 min was set around the retention time of each PA and the cycle time was fixed at 500 ms. To increase signal intensity, a ΔEMV setting of + 200 V was employed. To avoid matrix effects, an external matrix-matched calibration employing 7-point calibration curves was performed and resulted in limits of detection (LOD) and limits of quantification (LOQ) as shown in Table 1. LOD and LOQ were determined according to DIN 32,645 by calculating the process standard deviation s_{x0} of the linear calibration curve, whereas the LOD was defined as the 3.6-fold value of s_{x0} and the LOQ as the 10.8-fold value of s_{x0}, respectively (DIN 32654, 2008). Values below the LOQ are given as not detectable. The validated linear working range of the method was between 0.1 and 40 ng/mL. Sample solutions exceeding this calibration range were diluted accordingly. For method validation, recovery values were determined at low and high spiking levels by spiking a blank fennel matrix with known amounts of each analyzed PA(NO) at levels of 8 and 80 µg/kg plant material in quintuplicate, respectively. For verification purposes of this pollen study, recovery values were determined by spiking a blank pollen matrix with known amounts of each analyzed PA(NO) at a medium to low PA level of 20 µg/kg pollen (Table 1). Throughout the study, sample results were not corrected for recovery.

Palynological analysis

The samples were brought up to room temperature, homogenized with a mortar. An aliquot of 100 mg homogenized bee pollen was weighed into a 50-mL tube (Buddeberg, Mannheim, Germany) and mixed with 10 mL demineralized water. After adding a drop of dish soap, the mixture was shaken by hand for 1 min. An aliquot of 10 µL was transferred to an object carrier, dried for 30 min, and covered with Kaiser's glycerol gelatin for microscopy (Merck, Darmstadt, Germany). Three hundred pollen grains per sample were manually counted under a light microscope (10×40; VWR International, Darmstadt, Germany) which is considered representative for a pollen sample (Barth et al., 2012; Carpes et al., 2013; Morais et al., 2011). Due to the sample size of 57 pollen samples, 17,100 pollen grains were studied and subdivided into pollen from known PA producers and other plant families. Ten comparative samples were used to classify the pollen of PA-producing plants. The *Asteraceae* family includes many pollen types and each of them represents different plant species. However, pollen shape and size differ slightly from species to species within this family. For instance, the size of pollen was used to distinguish *Solidago* from *Senecio* pollen. Specifically, pollen sizes between 20 and 25 µm were assigned to pollen grains of the *Solidago*-type, which means all plants of *Asteraceae* which share similar shape and size of *Solidago* pollen cannot be differentiated. Therefore, *Eupatorium* sp. pollen were assigned to the group of *Solidago*-type. Pollen sizes > 30 µm (within the *Asteraceae* family) were considered to be of the *Senecio*-type. Using this classification scheme, *Petasites* and *Adenostyles* pollen belong to the *Senecio*-type. However, according to the literature, where measurements of six *Bidens* species and 20 *Senecio* species are listed, some *Bidens* species have similar pollen sizes as *Senecio* and vice versa (Beug, 2015). Hence, these two species could not be precisely distinguished by means of pollen sizes.

Results and discussion

Pyrrolizidine alkaloids detected by LC–MS/MS

In total, 52 of the 57 analyzed samples were detected with positive PA findings. Except for three samples without detectable PA and two samples with levels below LOQ, ΣPA concentrations ranged from 0.48 to 48,400 ng/g bee pollen (Table 2; Fig. 2). Extraordinarily high ΣPA concentrations in a few samples had a strong impact on the mean values. This can be seen from the fact that median and mean ΣPA concentrations of 44 and 2160 ng/g bee pollen in all samples, respectively, varied by two orders of magnitude. The median and mean ΣPA concentrations in the positive samples showed only minimally higher values (Table 2).

Furthermore, 24 of the 42 analyzed PA/PANO were detected in the 57 samples (Table S3). This variety included 13 basic structures which were predominantly present as PANO (96%) along with minor

Table 1 Analyzed substances with MRM details. LOD (ng/g), LOQ (ng/g) and recovery rate (%) as obtained by the laboratory (CVUA)

Pyrrolizidine alkaloid	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Quantifier	Collision energy (eV)	Cell accel. (V)	LOD (µg/kg)	LOQ (µg/kg)	Recovery rate (fennel matrix 8 µg/kg)	Recovery rate (fennel matrix 80 µg/kg)	Recovery rate (pollen matrix 20 µg/kg)
7-Acetylinter-medine	17.87	342.2	198.1		36	1	0.53	1.6	105%	106%	91%
		342.2	180.1		16	3					
		342.2	120.1	x	30	3					
7-Acetylinter-medine-N-oxide	19.28	358.2	214.1	x	30	4	0.40	1.2	99%	102%	92%
		358.2	180.1		32	5					
		358.2	137.1		34	1					
7-Acetyllycops-amine	18.30	342.2	198.1		32	4	0.24	0.71	106%	107%	71%
		342.2	180.1		16	5					
		342.2	120.1	x	30	1					
7-Acetyllycop-samine-N-oxide	19.63	358.2	214.1	x	32	1	0.75	2.3	101%	104%	88%
		358.2	180.1		32	1					
		358.2	137.1		38	1					
Echimidine	23.66	398.2	220.1		16	2	0.35	1.1	91%	98%	91%
		398.2	120.1	x	28	1					
		398.2	83.0		28	2					
Echimidine-N-oxide	23.97	414.2	254.1	x	36	2	0.32	0.97	90%	98%	94%
		414.2	220.1		34	3					
		414.2	137.1		40	1					
Echinatine (with rinder-ine)	7.84	300.2	156.1		28	5	0.09	0.26	99%	103%	90%
		300.2	138.1	x	24	1					
		300.2	94.1		50	3					
Echinatine-N-oxide	10.28	316.2	172.1	x	30	5	0.28	0.84	101%	100%	74%
		316.2	138.1		32	3					
		316.2	94.1		50	3					
Erucifoline	5.65	350.2	322.2		26	1	0.30	0.89	104%	113%	93%
		350.2	120.1	x	30	1					
		350.2	67.1		54	1					
Erucifoline-N-oxide	9.28	366.2	136.1	x	36	1	0.93	2.8	120%	117%	103%
		366.2	120.1		35	4					
		366.2	118.1		38	4					

Table 1 (continued)

Pyrrolizidine alkaloid	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Quantifier	Collision energy (eV)	Cell accel. (V)	LOD (µg/kg)	LOQ (µg/kg)	Recovery rate (fennel matrix 8 µg/kg)	Recovery rate (fennel matrix 80 µg/kg)	Recovery rate (pollen matrix 20 µg/kg)
Europine	8.00	330.2	156.1		34	4	0.14	0.42	104%	104%	91%
		330.2	138.1	x	22	4					
		330.2	120.1		42	4					
Europine-N-oxide	10.05	346.2	256.1		28	1	0.18	0.54	97%	97%	80%
		346.2	172.1	x	36	3					
		346.2	111.1		54	1					
Heliosupine	23.80	398.2	336.2		18	1	0.24	0.72	93%	100%	93%
		398.2	220.1		18	1					
		398.2	120.1	x	30	1					
Heliosupine-N-oxide	25.64	414.2	352.2		26	1	0.19	0.57	90%	96%	98%
		414.2	254.1	x	30	1					
		414.2	94.1		56	3					
Heliotrine	15.98	314.2	156.1		30	1	0.24	0.71	104%	102%	83%
		314.2	138.1	x	20	4					
		314.2	94.1		39	2					
Heliotrine-N-oxide	18.90	330.2	172.1	x	28	4	0.25	0.76	102%	99%	89%
		330.2	111.1		47	2					
		330.2	80.1		66	2					
Integerrimine	19.93	336.2	138.1		32	1	0.62	1.9	81%	92%	108%
		336.2	120.1	x	30	4					
		336.2	94.1		36	2					
Integerrimine-N-oxide	21.71	352.2	136.1		36	1	0.65	2.0	97%	101%	81%
		352.2	118.1		36	5					
		352.2	94.1	x	42	4					
Intermedine	7.02	300.2	156.1		27	4	0.16	0.47	95%	96%	87%
		300.2	138.1		20	2					
		300.2	94.1	x	28	1					
Intermedine-N-oxide	10.79	316.2	172.1		30	4	0.34	1.0	104%	96%	74%
		316.2	138.1	x	32	4					
		316.2	94.1		52	1					

Table 1 (continued)

Pyrrolizidine alkaloid	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Quantifier	Collision energy (eV)	Cell accel. (V)	LOD (µg/kg)	LOQ (µg/kg)	Recovery rate (fennel matrix 8 µg/kg)	Recovery rate (fennel matrix 80 µg/kg)	Recovery rate (pollen matrix 20 µg/kg)
Jacobine	7.35	352.2	280.2		24	1	0.75	2.2	112%	104%	88%
		352.2	155.1	x	30	3					
		352.2	77.1		56	1					
Jacobine-N-oxide	10.59	368.2	296.2	x	24	1	0.45	1.4	99%	101%	84%
		368.2	120.1		40	4					
		368.2	94.1		58	3					
Lasiocarpine	26.49	412.2	336.2		19	2	0.24	0.73	93%	102%	87%
		412.2	220.1		19	3					
		412.2	120.1	x	27	1					
Lasiocarpine-N-oxide	27.30	428.2	352.2		24	1	0.64	1.9	98%	99%	104%
		428.2	254.2	x	32	1					
		428.2	94.1		54	1					
Lycopsamine	7.54	300.2	156.1		32	1	0.13	0.40	94%	97%	88%
		300.2	138.1		22	1					
		300.2	94.1	x	29	1					
Lycopsamine-N-oxide	11.49	316.2	172.1	x	30	4	0.25	0.74	101%	98%	72%
		316.2	138.1		30	4					
		316.2	94.1		48	1					
Monocrotaline	3.33	326.2	280.2		24	1	0.16	0.48	107%	101%	108%
		326.2	194.1		31	4					
		326.2	120.1	x	42	1					
Monocrotaline-N-oxide	7.06	342.2	137.1	x	33	2	0.72	2.2	102%	98%	63%
		342.2	120.1		39	1					
		342.2	94.1		56	2					
Retrorsine	14.17	352.2	138.1		31	2	0.87	2.6	95%	99%	86%
		352.2	120.1	x	32	1					
		352.2	94.1		36	1					
Retrorsine-N-oxide	16.88	368.2	136.1		42	1	2.2	6.5	97%	97%	81%
		368.2	118.1		33	1					
		368.2	94.1	x	52	2					

Table 1 (continued)

Pyrrolizidine alkaloid	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Quantifier	Collision energy (eV)	Cell accel. (V)	LOD (µg/kg)	LOQ (µg/kg)	Recovery rate (fennel matrix 8 µg/kg)	Recovery rate (fennel matrix 80 µg/kg)	Recovery rate (pollen matrix 20 µg/kg)
Riddelliine	9.69	350.2	138.1		32	2	2.6	7.9	101%	97%	89%
		350.2	120.1	x	32	3					
		350.2	94.1		44	2					
Riddelliine-N-oxide	12.33	366.2	136.1		38	2	3.6	11	101%	98%	84%
		366.2	120.1	x	38	3					
		366.2	94.1		50	2					
Rinderine-N-oxide	9.98	316.2	172.1	x	32	3	0.15	0.46	94%	95%	78%
		316.2	111.1		48	2					
		316.2	94.1		48	2					
Senecionine	20.31	336.2	138.1		34	1	0.17	0.51	98%	100%	106%
		336.2	120.1	x	30	2					
		336.2	94.1		40	1					
Senecionine-N-oxide	21.88	352.2	136.1		40	1	0.23	0.70	96%	98%	81%
		352.2	118.1		38	1					
		352.2	94.1	x	51	1					
Seneciphylline	15.67	334.2	138.1		29	2	0.82	2.5	106%	100%	88%
		334.2	120.1	x	30	3					
		334.2	94.1		40	2					
Seneciphylline-N-oxide	19.01	350.2	136.1		37	2	0.49	1.5	101%	102%	119%
		350.2	120.1		40	2					
		350.2	94.1	x	52	3					
Senecivernine	19.45	336.2	308.1		30	1	0.52	1.6	100%	104%	88%
		336.2	138.1		34	1					
		336.2	120.1	x	32	3					
Senecivernine-N-oxide	21.12	352.2	136.1		40	1	0.40	1.2	98%	99%	90%
		352.2	118.1		46	1					
		352.2	94.1	x	43	4					
Senkirkine	23.78	366.2	168.1	x	32	5	1.3	3.8	64%	90%	89%
		366.2	150.1		28	4					
		366.2	122.1		32	5					

Table 1 (continued)

Pyrrolizidine alkaloid	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Quantifier	Collision energy (eV)	Cell accel. (V)	LOD (µg/kg)	LOQ (µg/kg)	Recovery rate (fennel matrix 8 µg/kg)	Recovery rate (fennel matrix 80 µg/kg)	Recovery rate (pollen matrix 20 µg/kg)
Trichodesmine	12.90	354.2	308.2		24	2 0.76		2.3	95%	101%	88%
		354.2	222.1	x	32	2					
		354.2	121.1		35	1					

contributions (4%) of all but two also as (free) PA (jacobine, retrorsine) and two of them as 7-acetyl-PA. As can be seen from Table 3, lycopsamine and intermedine are characteristic for plants of groups 2, 3, and 4, respectively, whereas plants of group 2 featured besides echimidine only lycopsamine and intermedine of the 42 PA included in the study.

However, commercial bee pollen samples mostly consist of one pool sample collected over the whole collection season (April to August), while our samples were pooled within a short period of ~ 14 days (rainy days excluded) in the end of July. Compared to our short sampling period during the blooming time of PA producers, the longer collection periods over 5 months in the literature study may have caused a dilution effect by the inclusion of daily bee pollen samples free of PA. Although palynological analysis only indicated 33 samples with PA-containing pollen (Fig. S2), chemical LC–MS/MS analysis verified PA in 52 of 57 pollen samples (91%) (Fig. 3; Table S3). This could be partly due to a high number of screened PA as well as low LOD values of ~0.1–3.5 ng/g bee pollen. For instance, six out of 39 bee pollen samples containing echinatine featured this alkaloid at levels < 1 ng/g bee pollen (Table S3).

Altogether, 16 bee pollen samples exceeded ΣPA concentration of > 1000 ng/g bee pollen. Similarly, seven individual PANO and one PA (echinatine/rinderine, both co-eluted) individually contributed > 1000 ng/g bee pollen to ΣPA (Fig. S3). It is noteworthy that for the highest contaminated sample, echinatine-NO (23,900 ng/g bee pollen) and rinderine-NO (19,000 ng/g bee pollen) dominated the sample with free echinatine/rinderine at ~ 1000 ng/g bee pollen (Fig. 2). Hence, free echinatine/rinderine represented only ~ 2% of the PANO level. Also, in other samples, free echinatine/rinderine reached only 1–7% of the corresponding PANO level (sum of echinatine-NO and rinderine-NO) (Table 4; Table S3). This further illustrated the predominant role of PANO compared to PA. Apart from this group, lycopsamine-NO, intermedine-NO, echimidine-NO, retrorsine-NO, and also senecivernine-NO exceeded the 1000 ng/g bee pollen level in one or two samples (Fig. S3).

Echinatine-NO ($n = 8$) and rinderine-NO ($n = 7$) not only show the highest frequency of > 1000 ng/g ΣPA concentrations, but both were usually detected with a very constant echinatine-NO/rinderine-NO (E/R) ratio of close to 1 (Fig. 4). This included

Table 2 Main results of pyrrolizidine alkaloids (PA) analysis in pollen samples from 57 locations in Southern Germany sampled in July 2019

Positive samples	Minimum ∑PA conc (ng/g)*	Maximum ∑PA conc (ng/g)	Mean ∑PA conc in pos. samples (ng/g)	Median ∑PA conc in pos. samples (ng/g)	Mean ∑PA conc in all samples (ng/g)	Median ∑PA conc in all samples (ng/g)
52	0.48	48,400	2370	93.5	2160	44

*Seven samples (9%) were < LOD

all samples at ΣPA > 500 ng/g bee pollen that featured a high content of rinderine-NO compared to echinatine-NO. This produced strong evidence that both PA were co-occurring in the contaminated pollen and therefore originated from the same plant source. Interestingly, 70% of the samples featured both echinatine-NO and rinderine-NO on a similar level (except 6 samples; Table 3). These samples featured pollen of *Solidago-/Bidens-/Eupatorium*-type. As mentioned before, these genera could not be distinguished by palynological analysis (see "Materials and methods"). Despite the equivocal palynological verification, coincidence of *Eupatorium* sp.–type PA and potential *Eupatorium* sp.–type pollen was striking.

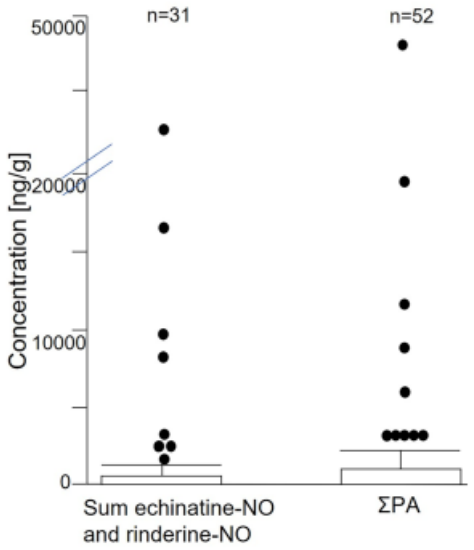

Fig. 2 Boxplot chart (created with JMP® pro 15.0) showing the distribution of the sum of echinatine-NO and rinderine-NO next to the ΣPA concentrations (ng/g)

Hence, we propose that echinatine-NO and rinderine-NO, present in about the same ratio, are suitable markers for *Eupatorium*-type PA (Table 3, group 4). This classic distribution was modified by assigning echinatine-NO when predominant (absence of rinderine-NO) to group 2 (*Echium*-type) (Table 3). Likewise, both lycopsamine-NO and intermedine-NO are also belonging to groups 2, 3, and 4.

Co-occurrence of echinatine-NO and rinderine-NO (*Eupatorium*-type) was the common case (Fig. 4). Only sample #25 was highly contaminated with echimidine-NO (2290 ng ΣPA/g bee pollen), without remarkable amounts of rinderine-NO and other PANO characteristics for *Eupatorium*-type pollen (Fig. S4a, b). This pointed to the presence of *Echium*-type PA in sample #25, which was verified by palynological analysis. This was one of the few samples in which *Echium* sp. pollen was palynologically detected and also the one with the highest number of pollen of this kind (53 of 300 counts). However, high abundance of echimidine-NO in sample #25 also indicated contamination by *Borago* sp. pollen (3 counts) (Tables 3 and 4). One further sample (#31, two *Echium* sp. counts) featured echimidine-NO at 70 ng/g bee pollen. Extrapolation of the level in sample #31 from two to 53 pollen counts (70 ng/g multiplied by factor 26.5) would result in an echimidine-NO level of ~ 1860 ng/g bee pollen, which was very similar to the amount in sample #25. However, other samples with *Echium* sp. counts (#44, #31, #29, #55) were low in or did not feature echimidine(-NO). Apparently, this PA was unsuited as a reliable marker for *Echium* sp. and concentrations were comparably low (echivulgarine (-NO) was shown to be the main alkaloid for *Echium* sp. pollen (Dübecke et al., 2011)).

All our data supports that *Eupatorium* sp. (group 4) and not *Echium* sp. (group 2) was the predominant reason for high PA concentrations in the present samples, due to sample collection period end of July. However, as already discussed, the palynological detection of *Eupatorium* sp. was equivocal

Table 3 PA grouped to their botanical origin

Group	Pyrrolizidine alkaloid	Necine base	Esterification
Group 1 (*Senecio*-type)	Jacobine	Retronecine	Cyclic
	Retrorsine	Retronecine	Cyclic
	Seneciphylline	Retronecine	Cyclic
	Senecivernine	Retronecine	Cyclic
	Senecionine	Retronecine	Cyclic
	Erucifoline	Retronecine	Cyclic
	Integerrimine	Retronecine	Cyclic
Group 2 (*Echium*-type)	Echimidine	Retronecine	Di
	Lycopsamine	Retronecine	Mono
	Intermedine	Retronecine	Mono
Group 3 (*Borago*-type)	Lycopsamine	Retronecine	Mono
	Intermedine	Retronecine	Mono
Group 4 (*Eupatorium*-type)*	Lycopsamine	Retronecine	Mono
	Intermedine	Retronecine	Mono
	Echinatine with rinderine	Heliotridine	Mono

**Eupatorium* sp. pollen is determined within the group of *Solidago-/Bidens*-type pollen

but mostly corresponding pollen was present when LC–MS/MS data indicated its presence. Based on this approach, 12 of the bee pollen samples with high PA content were assigned to group 4 (Fig. 4). Furthermore, most of these samples also featured lycopsamine-NO at around 10% of the concentration of rinderine-NO (Fig. S4a). For this reason, two samples also exceeded lycopsamine-NO levels of 1000 ng/g bee pollen ΣPA (Fig. 2).

As discussed above, most highly contaminated samples showed an E/R ratio close to 1 (Fig. 4). However, sample #12 formed an exception because

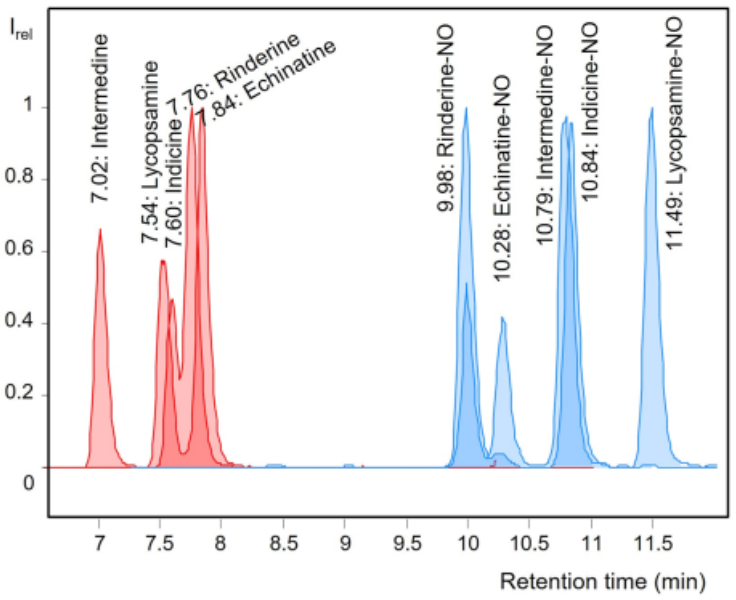

Fig. 3 LC–MS/MS chromatogram (excerpt) showing the elution order of diastereomeric PA and PANO. Overlay of a pollen matrix sample spiked with PA calibration mix containing 42 PA(NO) and a standard solution containing rinderine, indicine, and their N-oxides

Table 4 PA pattern in pollen samples > 500 ng/g ΣPA compared to pollen counts

Nr	Concentration PA (ng/g)							ΣPA [ng/g]	Counted pollen			
	Senecio-type	Echimidine (-NO)	Echinatine (-NO)	Lycopsamine-NO	Intermedine-NO	Rinderine-NO			*Borago* sp.	*Echium* sp.	*Senecio* sp.	*Solidago/Bidens-Eupatorium*-type
47	110	0	24,900	2420	1920	19,000		48,400			97	37
30	65	0	9070	1220	1030	8040		19,400		20	10	
53	0	1	4550	690	955	5400		11,600				6
1	0	0	5000	305	170	3350		8830				6
43	9	0	3330	455	280	1900		5960			14	1
12	2880	3	390	30	10	80		3390				
45	5	0	1590	190	190	1220		3190			2	3
41	1540	0	715	120	120	665		3160			1	
44	1	0	1180	70	150	1740		3140		1		
15	410	0	1600	110	55	770		2950			2	164
25	2	2290	0	5	0	0		2300	3	53		
56	0	6	860	130	105	830		1930				9
16	0	0	760	205	105	360		1430				1
31	0	70	500	55	45	390		1060		2		
29	625	0	230	20	10	170		1050		1	1	2
14	25	0	570	115	60	260		1030			3	12
13	0	0	355	50	25	220		650				6
48	2	0	260	15	15	330		620				1
37	0	5	235	40	40	260		570				
55	420	0	100	4	1	30		550		1		1

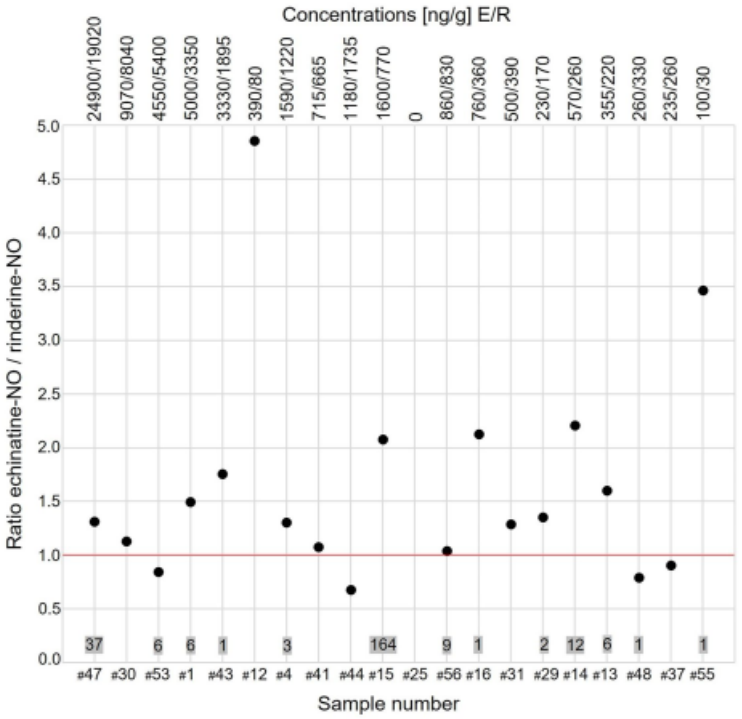

Fig. 4 Point chart showing the ratio between echinatine (-NO) and rinderine-NO (E/R) with concentrations (ng/g) at the top and pollen counts of *Solidago-/Bidens-/Eupatorium*-type pollen at the bottom

it was richer in echinatine-NO (E/R ~ 4) and did not feature *Echium* sp. pollen. Instead, the *Senecio*-type PA (including senecivernine-NO and retrorsine-NO) were detected at much higher abundance and contributed the most with 2880 ng/g bee pollen to the considerably high load of PA (ΣPA 3390 ng/g bee pollen). However, pollen of *Senecio* sp. could not be detected either in this sample (Table 4). Also, both PA did not play any noticeable role in other pollen samples (Table S4). Furthermore, one sample (#41) showed an E/R ratio close to 1 but also high abundance of *Senecio*-type PA (including senecionine-NO and seneciphylline-NO) with 1530 ng/g bee pollen (Table S4). In this sample, *Senecio* sp. pollen was counted once. Also sample #29 showed 50% content of *Senecio*-type PA, but only one *Senecio* sp. pollen was counted (Table 4). In further six samples (#47, #30, #43, #45, #15, #14), *Senecio* sp. pollen were counted, but the *Senecio*-type PA content was comparably low (< 13%) as compared to ΣPA (Table 4).

Accordingly, no connection could be made between frequency of pollen counts and concentrations of individual PA or ΣPA in the samples. As already discussed, the percentage of pollen from known PA-producing plants was only 3% in the samples (Fig. S2) while PA concentrations could be extremely high. Therefore, in agreement with the literature (Kast et al., 2019), palynological analysis was not suited to identify samples with high PA load. However, the high ΣPA levels in several samples were alarming. For this reason, a risk assessment was performed.

LC–MS/MS characteristics

The analyte spectrum (42 PA and PANO, see "Materials and methods") comprised a number of isobaric substances not only showing the same nominal mass but also exhibiting high structural similarities and thus sometimes even identical or at least very similar

MS/MS fragmentation patterns (Wuilloud et al., 2004). Therefore, chromatographic separation was granted special attention during method development to minimize possible co-elutions which bear the risk of erroneous peak identification. The use of a preferably long separation column in combination with a small particle size and a slow gradient elution profile (see "Materials and methods") enabled chromatographic separation or at least partial separation of problematic compound groups such as the diastereomeric PA intermedine, lycopsamine, echinatine, rinderine, and indicine, as well as their N-oxides (Fig. 3). Because of potential interferences with lycopsamine and intermedine-NO, indicine(-NO) was not routinely included in the calibration standards. Likewise, echinatine was chosen over rinderine to be included in the calibration set as both PA were only partially resolved. In contrast, the method allowed both rinderine-NO and echinatine-NO to be quantitated simultaneously. Thus, samples containing rinderine had their rinderine levels determined as echinatine.

The presented LC–MS/MS method has to be understood as a target analysis technique. Therefore, individual PA(NO) that were not included in the calibration set may go undetected by this method. This especially applies to PA that were not yet commercially available as standards, unless they happen to be isobaric to analytes that were already part of the target method. To overcome this issue, non-target analysis, e.g., employing high-resolution mass spectrometry (LC-HRMS), could be performed if the corresponding more expensive instrumentation was on hand. In non-target mode, detection is not limited to a certain set of reference standards and therefore potentially covers all PA, regardless of their availability. However, an important advantage of target analysis using LC–MS/MS is its outstanding sensitivity, which is often superior to the sensitivity achieved with LC-HRMS systems. As the safe determination even of low PA(NO) levels was considered crucial for the intended purpose, minor drawbacks of the target analysis technique were thus deemed acceptable. Furthermore, regarding the separation problem of diastereomeric analytes, even HRMS would not have presented an improvement as interfering compounds are typically isobaric and thus pose the same problem regardless of the mass spectrometric resolving power.

Palynological composition

Eighty-nine genera from 43 different plant families were identified by means of palynological inspection (Table S1), but only 3% (537 out of 17,100) of the inspected bee pollen grains could be traced back to PA-producing plants (Table 4; Fig. S2). Altogether, 33 samples (60%) contained pollen grains from known PA producers.

Pollen from *Echium* sp. were only present in seven of the present samples in this study (0.3% of all inspected pollen grains; Fig. S2). In addition, pollen of *Senecio* sp. were detected in 14 samples (1%) and *Borago* sp. in five samples (0.1%) (Fig. S2; Table S2). The highest share (1.7%) of PA-producing plants originated from *Solidago*-type and *Bidens*-type pollen (containing *Eupatorium* sp.) (23 samples) (Table S2).

Result comparison to previous studies

The results of palynological analysis in this study showed a clear dominance of *Eupatorium* sp. pollen in the samples from the end of July (Fig. S2) while a similar study from Switzerland reported a clear dominance of *Echium* sp. among PA producers in pollen collected in June and a dominance of *Eupatorium* sp. from mid-July to August (Kast et al., 2018). The detected *Eupatorium* sp. pollen and the collection time by the end of July can be compared between the samples from Germany and Switzerland (Kast et al., 2018).

Predominance of PANO over free bases (96% vs. 4%; Table S3) agreed with other studies of PA-contaminated plant pollen (Bodi et al., 2014; Boppré et al., 2005; Dübecke et al., 2011). Also, the maximum ΣPA concentration of 48,400 ng/g bee pollen (#47) was only slightly higher than the top concentration reported before in commercial pollen samples (38,000 ng ΣPA/g bee pollen, Dübecke et al. (2011)). However, the detection frequency of PA (91% positive findings) in the present samples was higher compared to 30–60% positive findings in previous commercial bee pollen investigations. This could be partly due to a higher number of screened PA than in other studies (Dübecke et al., 2011; Kempf et al., 2010) as well as slightly lower LOD values obtained in the present study. Typical LOD of individual PA in other studies were 1–10 ng/g bee pollen (Dübecke et al., 2011; Kast et al., 2018; Kempf et al., 2010)

compared to ~0.1–3.5 ng/g bee pollen in the present study.

Kast et al. (2018) analyzed one daily bee pollen sample on most of the weeks throughout the summers of 2012–2014 in Switzerland (one daily sample per week between April and September and flower heads from the neighborhood). This time series also indicated highest ΣPA concentrations in "*Eupatorium*-type PA" pollen samples collected at the end of July and the beginning of August (Kast et al., 2018). However, there was one remarkable difference between the results of Kast et al. (2018) and the present study. Although ΣPA concentrations were comparable between both studies, the PA pattern of *Eupatorium*-type pollen reported by Kast et al. (2018) was dominated by intermedine-NO and lycopsamine-NO. By contrast, both PANO were low concentrated in our samples which in turn were dominated by echinatine-NO and rinderine-NO (Table 4). Although all four PANO are known to occur in pollen of *Eupatorium* sp. plants (Roeder, 1995), the strikingly different PA patterns in the two studies from closely related regions were surprising. Since all four compounds were quantified in our samples by means of authentic reference standards (see "Materials and methods"), erroneous peak assignments could be excluded in our study. Compared to that, only a few PANO and none of the four crucial PANO were available to Kast et al. (2018) as reference standards. At this point, it is important to note that LC–MS/MS separation of intermedine-NO, rinderine-NO, echinatine-NO, and lycopsamine-NO was reported to be challenging (Colegate et al., 2012). Given the fact that rinderine-NO and echinatine-NO were not available as reference standards and a very short column (50 × 2.1 mm) was used by Kast et al. (2018), we concluded that the colleagues actually quantified these two PANO but wrongly labeled them as intermedine-NO and lycopsamine-NO although co-elutions of stereoisomers were discussed by the authors. Accordingly, a better resolution by LC–MS/MS and, most importantly, the availability of more reference standards in the present study enabled us to solve this discrepancy. Under this prerequisite, the highest concentrations of *Eupatorium*-type PA of 12,600 ng/g bee pollen reported by Kast et al. (2018) was on a similar level as top concentrations of 24,000 ng/g echinatine-NO of our samples.

Risk assessment

To assess carcinogenic effects of PA, EFSA has established a reference point (EFSA, 2017), which results (by application of an MOE of 10,000) in a maximum recommended daily intake of 24 ng PA per kg BW and day for humans (BfR, 2020). Exposures below this dose are considered unlikely to cause deleterious effects. Typically, 5–10 g bee pollen (1–2 teaspoons) are consumed as food supplement per day. A general mean body weight of 75 kg and a daily intake of 10 g bee pollen result in a maximum recommended daily intake of 1800 ng ΣPA. Hence, all samples with ΣPA concentrations > 180 ng/g bee pollen exceeded the current dietary recommendations for adults. Accordingly, only 33 of the 57 bee pollen samples (58%) showed ΣPA concentrations below the target value. Namely, five samples featured no PA measurable concentration, 16 samples featured ΣPA levels between 0.5 and 10 ng/g, and 12 samples featured between 10 and 160 ng/g pollen. Two bee pollen samples of the latter group would already exceed the maximum recommended daily intake for children (10–11 years) due to the lower BW of 40 kg, resulting in a safe ΣPA level of only 100 ng/g bee pollen (Fig. 5) (GBE, 2017). By contrast, 24 samples (42%) exceeded the ΣPA target value of 180 ng/g pollen with eight samples between 170 and 1000 ng/g bee pollen. Remarkably, 13 samples were contaminated with 1000–10,000 ng ΣPA/g bee pollen and three samples even with > 10,000 ng ΣPA/g bee pollen which strongly exceeded the maximum recommended daily intake according to BfR based on a normal portion size (Fig. 5). This share was about twice as high as reported for commercial bee pollen samples from Switzerland, where one-third of commercial samples featured PA with seven samples exceeding the BfR recommendations with up to 1.185 ng/g ΣPA (Kast et al., 2018). However, the samples in our study were collected within 14 days in July, i.e., during the most prominent flowering period of PA-producing plants. In comparison, commercial samples are frequently pools of pollen harvested during several months, which may have a diluting effect on the PA pollen load. Hence, our study presents a worst-case scenario. However, this approach also made it possible to determine less abundant PA in the samples which might have been overlooked in other periods of the year. Moreover, 21% of the samples of

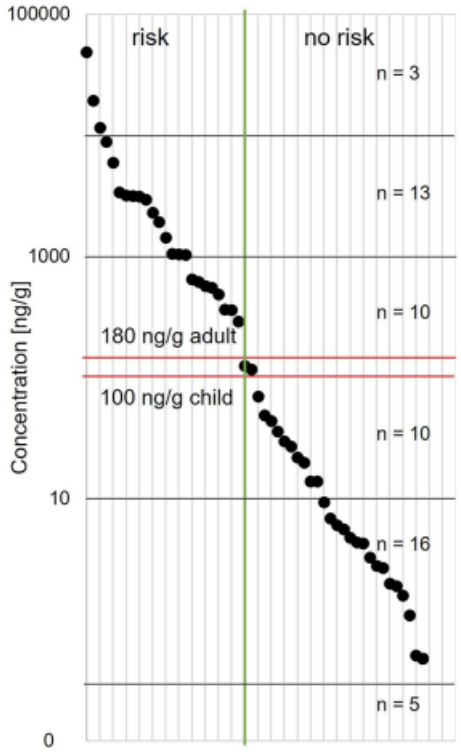

Fig. 5 Point chart showing the ΣPA concentrations (ng/g) with a potential risk according to the BfR recommendations

the present study showed 10 to 270 times higher ΣPA concentrations than recommended (BfR, 2020). Also, preliminary relative potency factors (REPs) are used to assess the risk of PA exposure, the toxic effects, and the potencies of various congeners. For open-chain and cyclic diesters with *7S* configuration (e.g., lasiocarpine), REP values of 1.0 were calculated, and for monoesters with *7S* configuration (e.g., echinatine) values of 0.3, while open-chain diesters with *7R* configuration assigned values of 0.1 (e.g., echimidine) and *7R* monoesters 0.01 (e.g., intermedine) (Merz & Schrenk, 2016). The values of the N-oxides are related to the corresponding PA. Results of this study showed especially high values of echinatine-NO, rinderine-NO, and lycopsamine-NO where the REP values are comparably low. PA with high REP values could only be detected occasionally in high concentrations. In summary, the consumption of highly PA-contaminated bee pollen on a regular basis may pose an increased risk of cancer or liver disease and is therefore discouraged. In general, PA exposure should be kept as low as possible, especially since the total exposure toward PA may be influenced by additional PA sources in human nutrition such as herbal teas, spices, or honey.

Conclusions

Ninety-one percent of the 57 bee pollen samples from different locations in Baden-Wuerttemberg featured PA with maximum ΣPA concentrations of 48,400 ng ΣPA/g bee pollen. The vast majority (96%) originated from PANO. Echinatine-NO and rinderine-NO showed highest concentrations of up to 23,900 ng/g bee pollen and were the most frequently detected PA in the samples collected at the end of July. About every third sample (20 out of 57 samples) exhibited ΣPA levels above the future legal maximum level of 500 ng/g pollen. In total, 24 pollen samples exceeded the BfR recommendation of 180 ng/g PA. Palynological analysis was found to be inappropriate to identify samples with high PA contamination.

The results of our study clearly demonstrate that almost half of the analyzed pollen samples from Southern Germany in July contained concentrations above the BfR recommendations. The occurrence of such high ΣPA concentrations could probably be minimized by stopping collecting bee pollen at the end of June (Kast et al., 2018). Furthermore, beekeepers in Southern Germany should avoid as much as possible the presence of *Eupatorium* sp. in the neighborhood around their hives. Bee pollen samples from Switzerland indicated no risk of contamination by pollen from *Eupatorium* sp. in months before July (Kast et al., 2018). Yet, further studies have to be conducted to check if this scenario can be assumed for Southern Germany as well. Also, commercial pollen samples from Germany should be examined for PA as they could consist pollen sampled in different months. At this point, bee pollen samples collected in July and before in Southern Germany should not be marketed or consumed without previous examination by means of LC–MS/MS analysis. The method applied in this study is suggested for application in order to establish a more robust assessment of PA content.

Acknowledgements The authors thank the volunteer beekeepers for collecting the pollen samples. Also, the authors are thankful for critical reading of the manuscript by PD. Dr. Peter Rosenkranz.

Author contribution Carolin Friedle: conceptualization, methodology, resources, data curation, formal analysis, visualization, writing – original draft, writing – review and editing; Thomas Kapp: methodology, data curation, analysis, writing – review and editing; Klaus Wallner: conceptualization, data curation, funding acquisition, supervision, writing – review and editing; Raghdan Alkattea: data curation, formal analysis, writing – review and editing; Walter Vetter: conceptualization, data curation, supervision, writing – review and editing.

Funding Open Access funding enabled and organized by Projekt DEAL. This study was supported by the Ministry of Rural Areas and Consumer Protection Baden-Wuerttemberg.

Availability of data and materials The authors confirm the data generated or analyzed during this study are included in this published article and its supplementary files. All datasets are also available from the corresponding author on reasonable request.

Declarations

Ethics approval The authors confirm that ethical standards were addressed.

Consent to participate The authors confirm the volunteer's declaration of consent.

Consent for publication The authors confirm the volunteer's consent for publication. The map generated by the State Office for Geoinformation and Rural Development Baden-Wuerttemberg and the Environmental Information System (UIS) of the LUBW State Institute for Environment Baden-Wuerttemberg have been given permission to publish by mentioning them as basic data.

Competing interests The authors declare no competing interests.

References

Avni, D., Hendriksma, H. P., Dag, A., Uni, Z., & Shafir, S. (2014). Nutritional aspects of honey bee-collected pollen and constraints on colony development in the eastern Mediterranean. *Journal of Insect Physiology, 69*, 65–73. https://doi.org/10.1016/j.jinsphys.2014.07.001

Barth, O. M., Freitas, A., & Alemeida-Muradian, L. B. (2012). 4. Palynological analysis of Brazilian stingless bee pot-honey: Stingless bees process honey and pollen on cerumen pots. *EN Vit P & Roubik DW*, 1–8.

Beug, H. J. (2015). *Leitfaden der Pollenbestimmung für Mitteleuropa und angrenzende Gebiete* (2nd ed.). Dr. Friedrich Pfeil.

BfR. (2013). Pyrrolizidine alkaloids in herbal teas and teas - BfR opinion 018/2013 of July 5, 2013: Pyrrolizidinalkaloide in Kräutertees und Tees - Stellungnahme 018/2013 des BfR vom 5. Juli 2013.

BfR. (2014). Bestimmung von Pyrrolizidinalkaloiden (PA) in Pflanzenmaterial mittels SPE-LC-MS/MS: Methodenbeschreibung: Determination of pyrrolizidine alkaloids (PA) in plant material using SPE-LC-MS/MS: Method Description.

BfR. (2020). *Updated risk assessment on the content of 1,2-unsaturated pyrrolizidine alkaloids (PA) in food: BfR Opinion No. 026/2020 of June 17, 2020: Updated risk assessment on the content of 1,2-unsaturated pyrrolizidine alkaloids (PA) in food: BfR Opinion No. 026/2020 of June 17, 2020 Aktualisierte Risikobewertung zu Gehalten an 1,2-ungesättigten Pyrrolizidinalkaloiden (PA) in Lebensmitteln: Stellungnahme Nr. 026/2020 des BfR vom 17. Juni 2020*. https://www.bfr.bund.de/cm/343/aktualisierte-risikobewertung-zu-gehalten-an-1-2-ungesaettigten-pyrrolizidinalkaloiden-pa-in-lebensmitteln.pdf. Accessed 22 Jan 2021.

Bodi, D., Ronczka, S., Gottschalk, C., Behr, N., Skibba, A., Wagner, M., et al. (2014). Determination of pyrrolizidine alkaloids in tea, herbal drugs and honey. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment, 31*, 1886–1895. https://doi.org/10.1080/19440049.2014.964337

Böhme, F., Bischoff, G., Zebitz, C. P. W., Rosenkranz, P., & Wallner, K. (2018). Pesticide residue survey of pollen loads collected by honeybees (*Apis mellifera*) in daily intervals at three agricultural sites in South Germany. *PLoS One, 13*, e0199995. https://doi.org/10.1371/journal.pone.0199995

Boppré, M., Colegate, S. M., & Edgar, J. A. (2005). Pyrrolizidine alkaloids of Echium vulgare honey found in pure pollen. *Journal of Agricultural and Food Chemistry, 53*, 594–600. https://doi.org/10.1021/jf0484531

Carpes, S. T., de Alencar, S. M., Cabral, I., Oldoni, T., Mourão, G. B., Haminiuk, C., et al. (2013). Polyphenols and palynological origin of bee pollen of *Apis mellifera* L.

from Brazil. Characterization of polyphenols of bee pollen. *CyTA - Journal of Food, 11*, 150–161. https://doi.org/10.1080/19476337.2012.711776

Colegate, S. M., Stegelmeier, B. L., & Edgar, J. A. (2012). Dietary exposure of livestock and humans to hepatotoxic natural products. In J. Fink-Gremmels (Ed.), *Animal feed contamination: Effects on livestock and food safety* (pp. 352–382, Woodhead Publishing series in food science, technology and nutrition, no. 215). Cambridge, UK, Philadelphia: Woodhead Publishing Limited.

DIN 32654. (2008). Chemical analysis - Decision limit, detection limit and determination limit under repeatability conditions - Terms, methods, evaluation. Beug Verlag, Berlin. https://doi.org/10.31030/1465413

Drummond, F. A., Ballman, E. S., Eitzer, B. D., Du Clos, B., & Dill, J. (2018). Exposure of honey bee (*Apis mellifera* L.) colonies to pesticides in pollen, A statewide assessment in Maine. *Environmental Entomology, 47*, 378–387. https://doi.org/10.1093/ee/nvy023

Dübecke, A., Beckh, G., & Lüllmann, C. (2011). Pyrrolizidine alkaloids in honey and bee pollen. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment, 28*, 348–358. https://doi.org/10.1080/19440049.2010.541594

Edgar, J. A., Colegate, S. M., Boppré, M., & Molyneux, R. J. (2011). Pyrrolizidine alkaloids in food: A spectrum of potential health consequences. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment, 28*, 308–324. https://doi.org/10.1080/19440049.2010.547520

EFSA. (2011). Scientific opinion on pyrrolizidine alkaloids in food and feed. *EFSA Journal*. https://doi.org/10.2903/j.efsa.2011.2406

EFSA. (2017). Risks for human health related to the presence of pyrrolizidine alkaloids in honey, tea, herbal infusions and food supplements. *EFSA Journal. European Food Safety Authority, 15*, e04908. https://doi.org/10.2903/j.efsa.2017.4908

European Commission. (2020). COMMISSION REGULATION (EU) 2020/2040 of 11 December 2020 amending Regulation (EC) No 1881/2006 as regards maximum levels of pyrrolizidine alkaloids in certain foodstuffs.

Feás, X., Vázquez-Tato, M. P., Estevinho, L., Seijas, J. A., & Iglesias, A. (2012). Organic bee pollen: Botanical origin, nutritional value, bioactive compounds, antioxidant activity and microbiological quality. *Molecules (Basel, Switzerland), 17*, 8359–8377. https://doi.org/10.3390/molecules17078359

Federal Institute for Pharmaceuticals and Medical Products. (2016). Announcement to check the content of pyrrolizidine alkaloids to ensure the quality and safety of medicinal products that contain herbal substances or herbal preparations or homeopathic preparations made from herbal raw materials as active ingredients: Bekanntmachung zur Prüfung des Gehalts an Pyrrolizidinalkaloiden zur Sicherstellung der Qualität und Unbedenklichkeit von Arzneimitteln, die pflanzliche Stoffe bzw. pflanzliche Zubereitungen oder homöopathische Zubereitungen aus pflanzlichen Ausgangsstoffen als Wirkstoffe enthalten.

Food and Drug Administration. (2001). Safety alerts & advisories - FDA advises dietary supplement manufacturers to remove comfrey products from the market. Center for Food Safety and Applied Nutrition. https://www.fda.gov/food/guidance-documents-regulatory-information-topic-food-and-dietary-supplements/dietary-supplements-guidance-documents-regulatory-information. Accessed 11 Mar 2021.

Friedle, C., Wallner, K., Rosenkranz, P., Martens, D., & Vetter, W. (2021). Pesticide residues in daily bee pollen samples (April–July) from an intensive agricultural region in Southern Germany. *Environmental Science and Pollution Research*. https://doi.org/10.1007/s11356-020-12318-2

GBE. (2017). Average body size of the population (height in m, weight in kg). Structural characteristics: Years, Germany, age, gender: Durchschnittliche Körpermaße der Bevölkerung (Größe in m, Gewicht in kg). Gliederungsmerkmale: Jahre, Deutschland, Alter, Geschlecht. https://www.gbe-bund.de/gbe/pkg_isgbe5.prc_menu_olap?p_uid=gast&p_aid=54890293&p_sprache=D&p_help=3&p_indnr=223&p_indsp=&p_ityp=H&p_fid=. Accessed 14 Apr 2021.

Gottschalk, C., Huckauf, A., Dübecke, A., Kaltner, F., Zimmermann, M., Rahaus, I., et al. (2018). Uncertainties in the determination of pyrrolizidine alkaloid levels in naturally contaminated honeys and comparison of results obtained by different analytical approaches. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment, 35*, 1366–1383. https://doi.org/10.1080/19440049.2018.1468929

Hartmann, T., & Toppel, G. (1987). Senecionine n-oxide, the primary product of pyrrolizidine alkaloid biosynthesis in root cultures of Senecio vulgaris. *Phytochemistry, 26*, 1639–1643. https://doi.org/10.1016/S0031-9422(00)82261-X

Kakar, F., Akbarian, Z., Leslie, T., Mustafa, M. L., Watson, J., van Egmond, H. P., et al. (2010). An outbreak of hepatic veno-occlusive disease in Western Afghanistan associated with exposure to wheat flour contaminated with pyrrolizidine alkaloids. *Journal of Toxicology, 2010*, 313280. https://doi.org/10.1155/2010/313280

Kaltner, F., Rychlik, M., Gareis, M., & Gottschalk, C. (2020). Occurrence and risk assessment of pyrrolizidine alkaloids in spices and culinary herbs from various geographical origins. *Toxins, 12*, 155. https://doi.org/10.3390/toxins12030155

Kast, C., Kilchenmann, V., Reinhard, H., Bieri, K., & Zoller, O. (2019). Pyrrolizidine alkaloids: The botanical origin of pollen collected during the flowering period of Echium vulgare and the stability of pyrrolizidine alkaloids in bee bread. *Molecules (Basel, Switzerland)*. https://doi.org/10.3390/molecules24122214

Kast, C., Kilchenmann, V., Reinhard, H., Droz, B., Lucchetti, M. A., Dübecke, A., et al. (2018). Chemical fingerprinting identifies Echium vulgare, Eupatorium cannabinum and Senecio spp. as plant species mainly responsible for pyrrolizidine alkaloids in bee-collected pollen. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment, 35*, 316–327. https://doi.org/10.1080/19440049.2017.1378443

Kempf, M., Heil, S., Hasslauer, I., Schmidt, L., von der Ohe, K., Theuring, C., et al. (2010). Pyrrolizidine alkaloids in pollen and pollen products. *Molecular Nutrition & Food Research, 54*, 292–300. https://doi.org/10.1002/mnfr.200900289

Kempf, M., Wittig, M., Schönfeld, K., Cramer, L., Schreier, P., & Beuerle, T. (2011). Pyrrolizidine alkaloids in food: Downstream contamination in the food chain caused by honey and pollen. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment, 28*, 325–331. https://doi.org/10.1080/19440049.2010.521771

Mărgăoan, R., Mărghitaş, L. A., Dezmirean, D. S., Dulf, F. V., Bunea, A., Socaci, S. A., et al. (2014). Predominant and secondary pollen botanical origins influence the carotenoid and fatty acid profile in fresh honeybee-collected pollen. *Journal of Agricultural and Food Chemistry, 62*, 6306–6316. https://doi.org/10.1021/jf5020318

Martinello, M., Cristofoli, C., Gallina, A., & Mutinelli, F. (2014). Easy and rapid method for the quantitative determination of pyrrolizidine alkaloids in honey by ultra performance liquid chromatography-mass spectrometry: An evaluation in commercial honey. *Food Control, 37*, 146–152. https://doi.org/10.1016/j.foodcont.2013.09.037

Merz, K. -H., & Schrenk, D. (2016). Interim relative potency factors for the toxicological risk assessment of pyrrolizidine alkaloids in food and herbal medicines. *Toxicology Letters, 263*, 44–57. https://doi.org/10.1016/j.toxlet.2016.05.002

Morais, M., Moreira, L., Feás, X., & Estevinho, L. M. (2011). Honeybee-collected pollen from five Portuguese Natural Parks: Palynological origin, phenolic content, antioxidant properties and antimicrobial activity. *Food and Chemical Toxicology : An International Journal Published for the British Industrial Biological Research Association, 49*, 1096–1101. https://doi.org/10.1016/j.fct.2011.01.020

Orantes-Bermejo, F. J., Serra Bonvehí, J., Gómez-Pajuelo, A., Megías, M., & Torres, C. (2013). Pyrrolizidine alkaloids: Their occurrence in Spanish honey collected from purple viper's bugloss (Echium spp.). *Food Additives & Contaminants. Part A Chemistry, Analysis, Control, Exposure & Risk Assessment, 30*, 1799–1806. https://doi.org/10.1080/19440049.2013.817686

Roeder, E. (1995). Medicinal plants in Europe containing pyrrolizidine alkaloids. *Die Pharmazie, 50*(2), 83–98.

Smith, L. W., & Culvenor, C. C. J. (1981). Plant sources of hepatotoxic pyrrolizidine alkaloids. *Journal of Natural Products, 44*(2), 129–152.

Taha, E. -K. A., Al-Kahtani, S., & Taha, R. (2019). Protein content and amino acids composition of bee-pollens from major floral sources in Al-Ahsa, eastern Saudi Arabia. *Saudi Journal of Biological Sciences, 26*, 232–237. https://doi.org/10.1016/j.sjbs.2017.06.003

Traynor, K. S., Pettis, J. S., Tarpy, D. R., Mullin, C. A., Frazier, J. L., Frazier, M., et al. (2016). In-hive pesticide exposome: Assessing risks to migratory honey bees from in-hive pesticide contamination in the Eastern United States. *Scientific Reports, 6*, 33207. https://doi.org/10.1038/srep33207

Wuilloud, J. C. A., Gratze, S. R., Gamble, B. M., & Wolnik, K. A. (2004). Simultaneous analysis of hepatotoxic pyrrolizidine alkaloids and N-oxides in comfrey root by LC-ion trap mass spectrometry. *The Analyst, 129*, 150–156. https://doi.org/10.1039/b311030c

4 Diskussion

In der hier dargestellten Arbeit wurden zum ersten Mal detaillierte Untersuchungen von Blütenpollen aus Baden-Württemberg durchgeführt. Erstmals wurden in täglich gesammelten Pollenproben einer Sammelsaison von April bis Ende Juli Pestizidrückstände untersucht. Insgesamt 102 Tagesproben konnten in einer intensiv bewirtschafteten Region gesammelt werden. Dabei lässt sich exemplarisch das Auftreten und die Beständigkeit von Pestiziden in Tagesproben darstellen. Zudem konnte das Wissen über die mikrobielle Zusammensetzung von Blütenpollen erweitert werden und erstmals Veränderungen der mikrobiellen Zusammensetzung unter bestimmten Lagerbedingungen außerhalb des Bienenstocks abgebildet werden. Neben Pestizidrückständen und mikrobiellen Einflüssen wurden in dieser Studie auch Pflanzeninhaltsstoffe wie PA untersucht, die den hohen Anteil von PA in saisonalem Blütenpollen, der im Juli gesammelt wird verdeutlichte. Dabei wurden erstmals 57 Mischproben aus Süddeutschland analysiert.

4.1 Diskussion der Methoden

4.1.1 Organisation und Sammeln der Pollenproben

Zur Organisation der Pollenproben wurde zunächst ein Sammelplan erstellt, in dem festgelegt wurde zu welchem Zeitpunkt und an welchen Standorten die Proben gesammelt werden sollten. Eine große Herausforderung stellte das Sammeln der Pollenproben in unterschiedlichen Regionen von Baden-Württemberg dar. Für diese Aufgabe wurden interessierte Imker*innen rekrutiert, die ihre Völker für diese Sammelversuche zur Verfügung stellten. Alle freiwilligen Imker*innen wurden mit kostenlosen Pollenfallen ausgestattet. Am Standort Oberteuringen, nähe Friedrichshafen, wurden die Daten der 102 täglich gesammelten Pollenproben erhoben. Die Ergebnisse wurden anhand eines Bienenvolkes an diesem Standort innerhalb einer

Sammelsaison im Jahr 2018 erfasst. Aufgrund der geringen Zahl von Völkern (n = 1) können die Ergebnisse nicht repräsentativ für andere intensiv bewirtschaftete Regionen in Betracht gezogen werden und müssen daher vorsichtig interpretiert werden (Chauzat et al. 2006; Drummond et al. 2018; Favaro et al. 2019).

Es sammeln ca. 20% der rund 30.000 Bienen aus einem Bienenvolk in einem Radius von bis zu 10 km^2 Pollen (Beekman und Ratnieks 2000; Klein et al. 2007). Diese hohe Zahl an Pollensammelnden Bienen wurde als angemessen betrachtet, um erste Erkenntnisse über das generelle Auftreten und die Beständigkeit von Pestiziden in täglich gesammelten Pollenproben zu erlangen. Dieses Szenario sollte exemplarisch zeigen, welchen Pestizidkonzentrationen Honigbienen durch das Sammeln von Pollen täglich und über eine ganze Sammelsaison ausgesetzt sind. Zudem wurde die Studie innerhalb eines Jahres durchgeführt. Aufgrund unterschiedlicher Klimabedingungen variieren die Blühzeiten von Pflanzen und das Auftreten von Schadorganismen jährlich (Sánchez-Pérez et al. 2014). Demnach variieren auch die Ausbringungszeiten der Wirkstoffe und Mengen der Spritzmaßnahmen an landwirtschaftlichen Kulturpflanzen in unterschiedlichen Jahren (Porter et al. 1991). Auch Klimabedingungen haben einen Einfluss auf das Flugverhalten der Bienen (Cobert et al. 1993). Während der Sammelsaison von April bis Ende Juli 2018 konnten nur wenige Regentage verzeichnet werden (DWD 2018), an denen die Bienen nicht ausfliegen und demnach keinen Pollen sammeln konnten. Folgende Jahre zeigten mehr Regentage in der Sammelsaison (DWD 2019, 2020), was die Flugmöglichkeiten und somit auch das Sammelverhalten der Honigbienen beeinflusst. Demnach können die Rückstände von Pestiziden in den gesammelten Tagesproben ebenfalls variieren. Weitere Studien mit einer höheren Anzahl an Völkern pro Standort in mehreren aufeinanderfolgenden Jahren müssen folglich durchgeführt werden, um repräsentative Aussagen über die Belastung von Pestizidrückständen in intensiv bewirtschafteten Regionen zu treffen.

Das gleiche Szenario findet sich bei der Untersuchung der mikrobiellen Zusammensetzung von frischen und gelagerten Blütenpollen wieder. In diesem Versuch wurden in zwei aufeinanderfolgenden Jahren an insgesamt drei Standorten in Baden-Württemberg Pollen gesammelt, im Juni 2018 an einem Standort in Hohenheim, im Juni 2019 in Forbach und Nürtingen. Die Proben wurden jeweils aus einem Volk an nur einem Tag im Juni frisch gesammelt und direkt für den Lagerversuch (siehe Diskussion der Ergebnisse) weiterverarbeitet. Demnach zeigen die Ergebnisse nur exemplarisch, welche Mikroorganismen in Pollen zu finden sind. Trotzdem geben die Ergebnisse nur erste Einblicke, weitere Studien sollten auch hier mit einer höheren Anzahl an Völkern und Standorten folgen, um repräsentative und fundierte Aussagen treffen zu können (Disayathanoowat et al. 2020) .

Um einen besseren Vergleich der Sammelzeiträume zu erlangen, wurden die Sammelperioden an die Blühzeiten bestimmter Pflanzen angepasst. Regionale Höhen- und daraus resultierende Klimaunterschiede sorgen dafür, dass Pflanzen nicht exakt zu denselben Zeiten im Jahr blühen (Ladányi et al. 2010). Zur Untersuchung von PA in saisonalen Pollenproben wurden Proben während der Blütenzeit von bekannten PA produzierenden Pflanzen *Echium sp.* (Natternkopf), *Boragp sp.* (Borretsch), *Eupatorium sp.* (Wasserdost) und *Senecio sp.* (Kreuzkräutern) gesammelt. Die Blühzeiten variieren zwischen Mai und September (Kast et al. 2018; Kast et al. 2019). Der Hauptteil der Blühzeit fällt in den Monat Juli. Deshalb wurden die Proben zur Untersuchung von PA Gehalten an allen beteiligten Standorten Ende Juli genommen.

4.1.2 Analyse der Pollenproben

Zur Aufarbeitung von Pestizidrückständen in unterschiedlichen Matrices wurde das einfache und schnelle QuEChERS-Verfahren modifiziert, dass international anerkannt ist und zur routinemäßigen Aufarbeitung verschiedener Probenmatrices verwendet wird (Anastassiades et al.

2003). Das QuEChERS-Verfahren wurde an die Labor- und Matrixbedingungen für die Aufarbeitung von Pollenproben an der Landesanstalt für Bienenkunde angepasst. Die Analyse von insgesamt 262 Wirkstoffen wurde im akkreditierten Analyselabor der landwirtschaftlichen Untersuchungs- und Forschungsanstalt in Speyer durchgeführt. Die Wiederfindungsrate aller Wirkstoffe wurde überprüft und mögliche Abweichungen diskutiert.

Die Analyse der Pollenproben zur Untersuchung der mikrobiellen Zusammensetzung stellte eine größere Herausforderung dar. Als Grundlage der Aufarbeitungsmethode wurde ein TRIzol Protokoll verwendet, dass in vergangen Studien schon zur Aufarbeitung der DNA von MO im Bienendarm verwendet wurde (D'Alvise et al. 2018; Seeburger et al. 2020). Verglichen mit den Ergebnissen der Bienenstudien konnten nur geringe Konzentrationen < 1 ng/µL an Pollen DNA extrahiert werden. Die extrahierten Proben wurden anschließend zur Analyse an spezialisierte Sequenzierlaboratorien versendet. Die Proben aus Hohenheim (2018) wurden bei Eurofins Genomics (Ebersberg) analysiert und bereits 2018 ausgewertet. Die Auswertung der Datensätze zeigte, dass die ITS1 Daten pilzunspezifisch präsentiert und die Pilze/Hefen lediglich in vier unterschiedliche Genera unterteilt wurden. Die Proben aus Forbach und Nürtingen (2019) wurden daraufhin bei StarSEQ (Mainz) analysiert. Nach intensiver Recherche und einem Vergleich der verwendeten Methoden wurde deutlich, dass Eurofins Genomics andere Primerpaare der ITS1 Region verwendete als StarSEQ. Die Primerpaare von Eurofins Genomics waren nicht pilzspezifisch, wohingegen StarSEQ pilzspezifische Primerpaare verwendete. Durch die finanzielle und materialtechnische Limitierung innerhalb des Projekts war eine Wiederholung der Analysen aus dem Jahr 2018 nicht möglich. Daraus resultieren die unterschiedlichen und nicht vergleichbare Ergebnisse der IST1 Region in den Proben. Folglich zeigen diese Ergebnisse deutlich, dass eine einheitliche Analyse von Proben notwendig ist, um Daten repräsentativ vergleichen zu können. In folgenden Studien sollte demnach exakt darauf geachtet werden, welche

Primer während der Analyse verwendet werden, um die Aussagekraft der erlangten Ergebnisse zu garantieren.

Die Analyse der Proben, die auf PA untersucht wurden, erfolgte im Chemischen und Veterinäruntersuchungsamt in Stuttgart. Verglichen mit vorangegangenen Studien wurden in der verwendeten Methode erstmals 42 verschiedene PA und PANO (N-Oxide) untersucht. Dabei ist die bemerkenswerte Trennleistung der Wirkstoffe zu beachten, die es ermöglicht hat, einzelne PA exakt voneinander zu trennen und bestimmen zu können. Die Bestimmung der einzelnen Pollen erfolgte mittels eines Lichtmikroskops, jedoch zeigte die palynologische Analyse der Pollenproben limitierende Faktoren: Einige Pollen unterschiedlicher Pflanzen weisen Ähnlichkeiten in Größe und Aussehen auf, deshalb konnten die Pollen, aufgrund ihres ähnlichen Aussehens nicht immer exakt voneinander unterschieden werden. Beispielsweise die Pollen der *Eupatorium sp*. wurden in der Solidago/Bidens-Gruppe mitbestimmt. Demnach konnten keine exakten Angaben gemacht werden, wie hoch der Anteil der *Eupatorium sp.* Pollen in den Proben war.

4.2 Diskussion der Ergebnisse

Verschiedene Qualitätsmerkmale von heimischem Blütenpollen aus Baden-Württemberg werden in der vorliegenden Arbeit erstmals in diesem Ausmaß untersucht. Bienenprodukte erlangen immer größere Bedeutung und ein stetig wachsendes Interesse in der Bevölkerung. Um der Verwendbarkeit als natürliches Nahrungsergänzungsmittel gerecht zu werden, ist es wichtig, dass Blütenpollen verschiedenen Qualitätsmerkmalen entsprechen.

4.2.1 Pestizide in Blütenpollen

Pflanzenschutzmittel belasten Blütenpollen und können daher die Qualität negativ beeinflussen. In Deutschland wird mehr als die Hälfte der Landfläche landwirtschaftlich genutzt, rund 90% wird dabei konventionell bewirtschaftet (Statistisches Bundesamt (Destatis) 2019; BMEL 2021). Um einen wirtschaftlichen Ertrag zu garantieren, werden sowohl ökologische als auch konventionelle Pflanzenschutzmittel in Kulturpflanzen eingesetzt. Zum Schutz von Kulturpflanzen vor Schädlingsbefall und Pilzwachstum, werden in Deutschland Sprühapplikationen von bis zu 15 verschiedenen Anwendungen in Obstgärten vom Frühling bis zum Spätsommer empfohlen (Wallner 2012). Doch nicht nur Kulturpflanzen sind Zielorganismen von dem Einsatz von PSM betroffen, bis zu 25% der ausgebrachten Mittel können durch Abdrift in der Umwelt verteilt werden und dabei in nicht zielgerichtete Vegetationen und Organismen gelangen (Aktar et al. 2009). Da Bestäuber wie Honigbienen und andere Insekten Nektar und Pollen nicht nur von Wildpflanzen, sondern auch von Kulturpflanzen in der Landwirtschaft sammeln, sind sie in einigen Regionen fortlaufend Spritzapplikationen oder Rückständen von Anwendungen ausgesetzt (David et al. 2016). Einige Studien zeigen bereits, dass vor allem von Honigbienen gesammelter Pollen und folglich auch das Bienenbrot stark mit Pestiziden belastet ist (Botías et al. 2016; Traynor et al. 2016; Favaro et al. 2019; Böhme et al. 2018; Deutsches Bienenmonitoring 2014-2019). Untersuchungen in den USA zeigen, dass Bienen in intensiv bewirtschafteten Regionen durch das Sammeln von Pollen Pestizidkonzentrationen von bis zu 99‘000 ng/g Pollen ausgesetzt sind (Mullin et al. 2010; Stoner und Eitzer 2013). Studien aus Deutschland konnten ebenfalls zeigen, dass Honigbienen in landwirtschaftlich bewirtschafteten Regionen Pollen mit enormen Konzentrationen von bis zu 7‘200 ng/g Pollen sammelten (Böhme et al. 2018). Bisher wurden jedoch lediglich Pollenproben wöchentlich oder monatlich auf Pestizidrückstände untersucht. Demnach fehlten detaillierte Informationen über die Entwicklung von Pestizidkonzentrationen

während einer Sammelsaison. Darüber hinaus waren die Häufigkeit und der zeitliche Verlauf von auftretenden Wirkstoffen weitgehend unbekannt.

Durch das tägliche Absammeln von Pollenhöschen vor dem Flugloch eines Bienenvolkes können die fehlenden Erkenntnisse über den Pestizideintrag in Pollen aus einer intensiv bewirtschafteten Region erworben werden (**Veröffentlichung 1**). Nach jetzigem Kenntnisstand gelang es erstmalig, umfassend die Entwicklung von Pestizidkonzentrationen in Pollen im Verlaufe einer Sammelsaison von April bis Juli darzustellen. Insgesamt 102 Pollenproben konnten für diese Untersuchung analysiert werden. Die Daten der Studie zeigen, dass in nahezu 90% aller analysierten Proben nachweisbare Konzentrationen von Pestizidrückständen gefunden werden konnten. Die aufgestellte Hypothese, dass in Pollenproben aus intensiv bewirtschafteten Regionen Pestizidrückstände über eine gesamte Sammelsaison von April bis Juli detektiert werden können, wurde damit bestätigt. Innerhalb dieser Proben wurden zwischen ein bis dreizehn unterschiedliche Wirkstoffe je Probe detektiert. Von 29 nachgewiesenen Pestiziden wurden 51% als Fungizide, 41% als Insektizide und 8% als Herbizide identifiziert. Der häufigste gefundene Wirkstoff war das Fungizid Trifloxystrobin mit 64 Detektionen in allen Proben. Die höchsten Konzentrationen konnten in Tagesproben von Ende April und Anfang Mai, sowie in der zweiten Junihälfte detektiert werden. Die höchste detektierbare Konzentration wies allerdings das Fungizid Tebuconazol mit 4530 ng/g Pollen auf. Die maximale Gesamtkonzentration an Pestiziden pro Probe konnte mit 8800 ng/g gemessen werden.

Auch wenn die gemessenen Konzentrationen hoch erscheinen, zeigten andere Studien weitaus höhere Wirkstoffkonzentrationen. Jährlich werden weltweit über 2,3 Millionen Tonnen an Pestiziden in der Landwirtschaft eingesetzt, alleine der Pestizidverbrauch der USA beträgt 25% (EPA 2007). Vorausgegangene Studien in den USA zeigen, dass Bestäuber wie Honigbienen in intensiv bewirtschafteten Regionen mit Extremwerten konfrontiert werden. Konzentrationen von bis zu 99‘000 ng/g stellen eine große Gefahr für Bestäuber und deren Brut dar. Es wurden

signifikante Einflüsse einiger Wirkstoffe zu einer akuten und subletalen Verringerung der Fitness von Honigbienen vermutet (Mullin et al. 2010). Um die Gefährdung von Honigbienen durch Pestizidrückstände in den Pollenproben zu bewerten, wurde ein sogenannter Pollen Hazard Quotient (PHQ) kalkuliert (Stoner und Eitzer 2013). Der PHQ wird berechnet, indem die detektierten Konzentrationen (ng/g) jedes nachgewiesenen Wirkstoffes durch den bekannten LD_{50}-Wert (Honigbiene oral; µg/Biene) dividiert wird (Pesticide Properties DataBase - PPDB 2010). Basierend auf dem täglichen Verzehr von bis zu 9,5 mg Bienenbrot durch eine Ammenbiene, wurde ein PHQ von ≥ 50 als kritisch für die Bienengesundheit angesehen (Böhme et al. 2018). In der vorgelegten Studie konnten PHQ Werte bis zu 170 in einzelnen Tagesproben kalkuliert werden. Vor allem Insektizide, die einen niedrigen LD_{50}-Wert nach Labortests aufweisen, zeigen schon bei geringen Pestizidkonzentrationen hohe PHQ Werte. In den USA wurden aufgrund sehr hoher Wirkstoffkonzentrationen und niedriger LD_{50}-Werte einiger Wirkstoffe, PHQ Werte bis zu 40‘000 kalkuliert (Stoner und Eitzer 2013). Diese außerordentlich hohen Werte könnten ein Grund für die schwerwiegenden jährlichen Verluste von bewirtschafteten Honigbienenvölkern in den USA sein (van Engelsdorp et al. 2012). Vergleichbare Ereignisse konnten in Deutschland bislang nicht beobachtet werden, trotzdem sind die Wirkstoffgehalte, vor allem in Betracht auf detektierte Insektizide nicht zu vernachlässigen.

Drei der nachgewiesenen Wirkstoffe (Diflubenzuron, Fenoxycarb und Spirodiclofen) wurden in dieser Studie als bienengefährlich eingestuft. Diflubenzuron und Fenoxycarb sind nicht für die Verwendung in blühende Pflanzen in Deutschland zugelassen (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit 2020). Insbesondere Fenoxycarb, ist bekannt durch negative Auswirkung auf die Bienenbrut (Czoppelt 1991; Aupinel et al. 2007). Mit einem detektierten Maximalwert von 370 ng Fenoxacarb / g Pollen in dieser Studie, wird der potentielle Schaden für Larven als gering eingeschätzt, jedoch sollten die möglichen Folgen der Existenz dieser bienengefährlichen Stoffe in den Proben nicht unterschätzt werden (Rortais et al. 2005).

Die Untersuchungen der 102 täglich gesammelten Pollenproben verdeutlichen, dass in fast jeder Tagesprobe Pestizidrückstände gefunden werden konnten. Der Standort in einer intensiv bewirtschafteten Obstanbau Region wurde gewählt, um exemplarisch ein „worst-case"-Szenario darzustellen. Der tägliche Verlauf der Wirkstoffkonzentrationen zeigt deutlich, dass viele Wirkstoffe gemeinsam eingesetzt werden. Die Fungizide Tebuconazol und Fluopyram weisen einen deutlichen Zusammenhang in der detektierten Konzentration innerhalb eines Zeitfensters von 10 Tagen auf. Mindestens ein Spritzmittel (Luna Experience) ist dazu passend bekannt, das in Deutschland vorwiegend im Obst- und Gemüseanbau verwendet wird (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit 2020). Auch weitere Wirkstoffe können in bis zu 10 aufeinanderfolgenden Tagen in den Proben gefunden werden.

Die Ergebnisse der ersten Veröffentlichung wurden bislang hinsichtlich Gefahren und Risiken für Honigbienen bewertet. Betrachtet man Blütenpollen als Nahrungsmittel für den menschlichen Verzehr, müssen andere Hilfsmittel zur Einschätzung des Risikos hinzugezogen werden. Nach Änderung der Verordnung VO (EG) 396/2005 Anhang 1 durch die Verordnung VO (EU) 2018/62 gelten für Blütenpollen zunächst keine Höchstmengengrenzen mehr. Ein allgemeiner Wert von 0.05 mg/kg (kann in Einzelfällen auch abweichen) gilt lediglich verbindlich für Honig (VO (EU) 2018/ 62). Blütenpollen ist per Rechtsverordnung auch mit weitaus höheren Pestizidrückständen in der EU verkehrsfähig und dadurch auch zum Verzehr geeignet. Deshalb sollte in Bezug auf mögliche resultierende Risiken eine toxikologische Bewertung der detektierten PSM in den Pollenproben folgen. Die EFSA gibt anhand von Risikountersuchungen und -bewertungen und eines miteinberechneten Schutzfaktors den sogenannten ADI-Wert vor. Der ADI- Wert (Acceptable Daily Intake) gibt die Menge eines Wirkstoffes an, die bei täglicher Aufnahme pro kg Körpergewicht zu keinen riskanten Folgen führt. In den analysierten Pollenproben zeigt Tebuconazol mit einem ADI von 0.03 mg/kg Körpergewicht pro Tag Höchstkonzentrationen von bis zu 4530 ng/g Pollen (EFSA 2010). Geht man von einer täglichen Aufnahme

von 1-2 Esslöffeln Pollen, das heißt maximal 10 g Pollen und einem durchschnittlichen Körpergewicht von 75 kg aus, dürften in den Pollenproben maximal 225‘000 ng/g Tebuconazol enthalten sein. Der errechnete Wert liegt weit über dem analysierten Höchstwert in den Proben. Aufgrund von Verdünnungseffekten durch Mischen der Tagesproben, kann davon ausgegangen werden, dass die analysierten Höchstwerte zusätzlich verringert werden. Folglich stellen die gefundenen Konzentrationen für den Menschen kein Gesundheitsrisiko dar und die analysierten Pollenproben können vom Menschen verzehrt werden.

4.2.2 Mikroorganismen in Blütenpollen

Neben Pflanzenschutzmitteln können auch Mikroorganismen (MO) die Qualität von Lebensmitteln stark beeinflussen. MO sind in der Umwelt wie in der Luft, im Wasser, im Boden, sowie in Pflanzen und Lebewesen zu finden (Zalar et al. 2007; Fridman et al. 2012). Sie können dabei sowohl nützliche als auch schädliche Wirkungen haben. Viele MO sind für unsere Ernährung unentbehrlich, denn sie werden zur Herstellung und Veredelung von Lebensmitteln eingesetzt, beispielsweise bei der Joghurt- oder Käseherstellung (Rehm 1980). Doch einige MO können durch ihre Anwesenheit einen negativen Einfluss auf das Lebensmittel, sowie auf die menschliche Gesundheit ausüben. Sie können zu dem Verderb von Lebensmitteln, sowie zu Lebensmittelvergiftungen und Lebensmittelinfektionen führen (BVL 2021c). Die meisten Mikroorganismen, die Lebensmittel verderben können benötigen Wasser, eine Energiequelle, sowie eine Stickstoffquelle, Vitamine und Mineralstoffe zum Leben (Keweloh 2016). Folglich sind rohe Lebensmittel mit einem hohen Wasser- und Proteingehalt wie beispielsweise Fleisch leicht verderblich (BVL 2021a). Frisch gesammelter Blütenpollen enthält vergleichbar zu Fleisch einen hohen Anteil an Wasser und Proteinen. Demnach ist Blütenpollen ein leichtverderbliches

Lebensmittel und muss nach der Ernte sorgfältig verarbeitet und gelagert werden, um ein Wachstum von Lebensmittelverderbenden MO zu verhindern.

Durch eine Lagerung unter verschiedenen Bedingungen von frisch gesammeltem Blütenpollen kann die Veränderung der präsenten Mikroorganismenzusammensetzung untersucht werden (**Veröffentlichung 2**). Erstmals wurde ein Lagerversuch außerhalb des Bienenstocks angesetzt, bei dem frisch gesammelter Blütenpollen unter kalten, moderaten und warm-feuchten Bedingungen gelagert wurde. An drei Standorten in Baden-Württemberg wurde im Juni 2018 und 2019 Blütenpollen frisch gesammelt und Lagerbedingungen außerhalb des Bienenstocks simuliert. Der Versuch soll zeigen, dass während einer warm-feuchten Lagerung von sieben Tagen die Mikroorganismenzusammensetzung in den Pollenproben negativ beeinflusst wird und eine Verarbeitung von Pollen nach der Ernte unabdingbar ist, um ein sicheres Lebensmittel zu erhalten. Die frischen und gelagerten Pollenproben wurden mittels einer palynologischen Analyse auf deren Pollenzusammensetzung untersucht, sowie mittels Amplikon-Sequenzierung auf Basis von DNA-Sequenzen der rRNA der V3-V4 Region und ITS1 Region die Zusammensetzung der MO untersucht. Die Daten zeigen, dass sich die Pollenproben je Standort und Jahr sowohl in deren Pollenzusammensetzung als auch in der MO-Zusammsetzung unterscheiden. Dominante Bakterien in den Proben vom Datenset 2019 waren *Lactobacillus, Pseudomonas, Rosenbergiella* und *Acinetobacter*. Als dominante Pilze und Hefen konnten *Cladosporium, Podosphaera, Mycosphaerella* und *Zygosaccharomyces* in den Proben detektiert werden. Die statistische Auswertung der Ergebnisse zeigt, dass der Standort sowohl bei der Bakterien- als auch der Pilzzusammensetzung einen signifikanten Einfluss hat (GLM; $p < 0.001$). Die Lagerbedingung zeigt nur bei *Lactobacillus* einen signifikanten Einfluss (Tukey; $p = 0.04$), wohingegen bei allen untersuchten Pilzen und Hefen (außer *Podospaera*) ein signifikanter Einfluss der Lagerbedingungen nachgewiesen werden konnte. *Zygosaccharomyces* und *Aspergillus* zeigen signifikante Unterschiede zwischen frisch – warmen, kalt – warmen, und Zimmertemperatur –

warmen Lagerbedingungen (Tukey, $p < 0.001$). Die größte Zunahme an proportionalem Anteil kann unter warmen Lagerbedingungen (30 °C, 75% Luftfeuchtigkeit) beobachtet werden.

Die Zusammensetzung der Bakterien zeigt unter definierten Lagerbedingungen nur geringe Veränderungen. Die dominierenden Bakterien wie *Pseudomonas* und *Rosenbergiella* sind auf einen pflanzlichen Ursprung zurückzuführen. Sie konnten bereits in pflanzlichem Material wie Nektar gefunden werden (Fridman et al. 2012; Halpern et al. 2013). Das Bakterium *Acinetobacter* findet sich in unterschiedlichen Medien, aber hauptsächlich ebenfalls in Pflanzen sowie im Darmtrakt von Honigbienen wieder (Fridman et al. 2012; Donkersley et al. 2018). Auch das Bakterium *Lactobacillus*, beispielsweise die Spezies *Lactobacillus kunkeii*, kann durch den Speichel von Honigbienen auf Blütenpollen übertragen worden sein (Anderson et al. 2014b; Donkersley et al. 2018). Bisher ist keines der dominierenden Bakterien bekannt dafür, die Qualität von Blütenpollen hinsichtlich der Verzehrbarkeit negativ zu beeinflussen.

Die Veränderung der Pilzzusammensetzung zeigte jedoch auffällige Ergebnisse. Während der Lagerung wiesen alle untersuchten Pilze und Hefen eine signifikante Änderung ihres Anteils in der Probe auf. *Cladosporium*, ein Pilz, der ubiquitär in der Umwelt wie in der Luft und im Boden vorhanden ist (Zalar et al. 2007), war in frisch gesammelten Pollenproben zu einem Anteil von bis zu 45% zu finden. Während der Lagerung von sieben Tagen nahm der Anteil allerdings deutlich ab. Die Pilze *Podoshaera* und *Mycosphaerella* zählen zu den Pflanzenpathogenen (Crous et al. 2006; Baiswar et al. 2010), deren Anteil in den Proben ebenfalls einen Rückgang während der Lagerung im Vergleich zu den frisch gesammelten Pollenproben zeigt. Während ein prozentualer Rückgang der oben genannten Pilze zu beobachten war, konnte jedoch besonders bei zwei Pilz- bzw. Hefegruppen ein deutlicher Zuwachs nach der warm-feuchten Lagerung vermerkt werden. *Zygosaccharomyces*, eine osmotolerante Hefe, dominierte deutlich in den warm gelagerten Pollenproben. Die Gattung ist ein bekannter Organismus, der vor allem in zuckerhaltigen Lebensmittel wie Fruchtsäften und Süßigkeiten zum Verderb durch

Fermentierung führen kann (Martorell et al. 2007). Die Hefe ist auch ein Mitglied der MO-Gemeinschaft im Darm von Honigbienen und kann daher durch die Zugabe von Bienenspeichel auf Blütenpollen übertragen werden (Yun et al. 2018). Durch die Produktion von Ethanol und Kohlendioxid aus Zucker können sensorische Eigenschaften wie Aroma und Farbe des Pollens durch die Fermentation negativ beeinflusst werden (Sperber und Doyle 2009). Um die Qualität von Aroma und Farbe des Pollens gewährleisten zu können, sollte das Wachstum von Hefen wie *Zygosaccharomyces* durch Einfrieren oder Kühlen verhindert werden. Die Ergebnisse der vorliegenden Studie zeigen, dass bei einer Lagerung unter 4°C nach sieben Tagen kein Wachstum an *Zygosaccharomyces* zu beobachten ist.

Auch der Anstieg der Pilzgattung *Aspergillus* unter warmen Lagerbedingungen muss im Hinblick auf Qualität von Blütenpollen und die menschliche Ernährung beobachtet werden. *Aspergillus* ist eine extrem halo- und osmotolerante Pilzgattung und enthält eine Reihe hoch mykotoxigener Arten (González et al. 2005). In Lebensmitteln kann Aspergillus durch sichtbares Wachstum von Schwarzschimmel zu Verfärbungen führen. Auch die Bildung von Mykotoxinen, die krebserzeugende Wirkungen und Schädigungen am Zentralnervensystem zeigen, können erheblichen Einfluss auf die menschliche Gesundheit ausüben (Sperber und Doyle 2009). Bereits eine vorangegangene Studie zeigte, dass Mykotoxine in verzehrfertigen Pollenproben aus Spanien gefunden werden können (González et al. 2005). Falsche Verarbeitung bezüglich Trocknung und Lagerung sowie nicht tägliche Ernte der Pollen wurden hier als Gründe aufgeführt. Die Ergebnisse der vorliegenden Studie unterstreichen diese Annahmen. Die Hypothese, dass Lagerbedingungen die Zusammensetzung der Mikroorganismen in Pollen beeinflussen, kann durch die dargestellten Ergebnisse bestätigt werden. Diese zeigen einen deutlichen Anstieg an Hefen und Pilzen, die mit großer Wahrscheinlichkeit die Ursache für einen Verderb von Blütenpollen verantwortlich sind. Daher ist es bei der Verarbeitung von frisch geerntetem Blütenpollen wichtig, das Wachstum von verderbenden MO zu verhindern.

4.2.3 Pyrrolizidinalkaloide in Blütenpollen

Auch wenn eine angemessene Verarbeitung erfolgt und Blütenpollen nach entsprechenden Analysen hinsichtlich hygienischer Aspekte und Rückstandsproblematik für den menschlichen Verzehr geeignet ist, können weitere Faktoren die Qualität des Produkts beeinflussen. Vergiftungsfälle durch PA sind bereits seit längerem bekannt, da PA durch die Aufnahme in der Leber zu hochreaktiven Pyrrolestern metabolisiert werden, die in der Lage sind, mit Nukleinsäuren und Proteinen zu reagieren und dadurch Leberschäden verursachen, aber auch kanzerogene Auswirkungen haben können (Edgar et al. 2011). Lebensmittel pflanzlichen Ursprungs wie Tee, Honig oder Blütenpollen gelten als Hauptquelle von PA und wurden deshalb schon in vielen Studien untersucht (Roeder 1995; Boppré et al. 2005; Kempf et al. 2011; Dübecke et al. 2011; Gottschalk et al. 2018; Kaltner et al. 2018). Neuste Studien aus der Schweiz zeigen, dass sowohl kommerzieller Blütenpollen als auch frisch gesammelter Pollen von Bienenvölkern enorme Mengen an PA enthalten können (Kast et al. 2018; Kast et al. 2019). Informationen von saisonal gesammeltem Blütenpollen aus Deutschland fehlen bislang.

Durch die Analyse von regionalem Blütenpollen aus Süddeutschland soll untersucht werden, ob PA in bedenklichen Mengen zu finden sind und welche Pflanzen dafür verantwortlich sein könnten (**Veröffentlichung 3**). Dazu wurden erstmals im Juli 2019 insgesamt 57 Pollenproben (14 Tagesproben zu einer Mischprobe vereint) von unterschiedlichen Standorten in Baden-Württemberg gesammelt und auf deren Pollenzusammensetzung sowie auf PA Rückstände untersucht. Mit einer Aufarbeitungs- sowie Messmethode der CVUA konnten die Proben auf 42 verschiedene PA/PANO untersucht werden. Insgesamt 25 PA konnten in 52 Proben detektiert werden, dabei konnten lediglich 5% der PA dem *Senecio-typ* zugeordnet werden, die anderen 95% der detektierten PA wurden von Pflanzen der Gattung *Echium sp*. und *Eupatorium sp*. produziert. Eine palynologische Analyse der Proben wies allerdings nur 3% an PA produzierenden Pflanzenpollen in den Proben auf. Ein Zusammenhang zwischen der Anzahl an

ausgezählten Pollenkörnern und der Höhe an detektieren PA Konzentrationen in den Proben konnte nicht nachgewiesen werden. Demnach lässt sich die Hypothese, dass sich PA Konzentrationen auf PA produzierende Pflanzen zurückführen lassen, nur bedingt bestätigen. Fast alle detektierten PA (96%) lagen als PANO in den Proben vor. Höchstkonzentrationen von > 1‘000 ng/g an Summe der PAs (ΣPA) konnten in 16 Proben gefunden werden, dabei zeigte eine Probe einen absoluten Höchstwert von 48‘400 ng/g ΣPA an. Das PANO Echinatin-N-oxid konnte dabei Höchstwerte von bis zu 23‘900 ng/g aufweisen. In vielen der Proben konnten allerdings nur sehr geringe Konzentrationen an PA gemessen werden, weshalb der Median der Proben bei lediglich 44 ng/g lag. Dessen ungeachtet sind die enorm hohen Konzentrationen an PA in den auffälligen Proben besonders zu betrachten.

Derzeit sind von der Europäischen Union keine Höchstwerte für PA in Lebensmitteln festgelegt. Nach einer Risikobewertung der Europäischen Behörde für Lebensmittelsicherheit (EFSA) wurde als Referenzdosis eine Konzentration von 237 µg pro kg Körpergewicht nach einem Tierversuch von Lasiocarpin und Riddelliin an Ratten abgeleitet (EFSA 2011). Unter der Berücksichtigung einer tolerierbaren Expositionsspanne von 10‘000 wurde für Menschen eine maximale Aufnahme von 0,024 µg PA pro kg Körpergewicht pro Tag berechnet (BfR 2020). Wird wieder von einer Aufnahme von 1-2 Esslöffeln Blütenpollen (10 g) pro Tag und einem durchschnittlichen Körpergewicht von 75 kg ausgegangen, kann ein tolerierbarer Wert von 180 ng PA pro g Pollen berechnet werden (GBE 2017). Von den Proben der vorgelegten Studie zeigen insgesamt 33 Pollenproben Konzentrationen unterhalb des tolerierbaren Wertes, wohingegen bei 24 Proben Konzentration weit über dem tolerierbaren Wert lagen. Unter Berücksichtigung der Angaben des Bundesamtes für Risikobewertung stellen 42% der untersuchten Proben ein erhöhtes Gesundheitsrisiko für den Menschen dar. Ferner zeigen 30% aller untersuchten Proben Konzentrationen mit bis zu 30-mal höheren Werten an. Auch in anderen Studien konnten solche Überschreitungen gezeigt werden (Kast et al. 2018), wobei die gefundenen

Konzentrationen in der vorliegenden Studie absolute Spitzenwerte darstellen und keine weitere Studie bekannt ist, in der solche Mengen an PA in Bienenprodukten wie Honig und Pollen gefunden werden konnten.

Die Ergebnisse dieser und vergangenen Studien zeigen deutlich, dass Pollenproben aus den Sommermonaten Juni, Juli und August hoch mit PAs belastet sein können (Kast et al. 2018; Kast et al. 2019). Auch wenn normalerweise Pollenproben über eine ganze Sammelsaison von April bis Juli gesammelt werden und demnach Verdünnungseffekte bei Mischung aller gesammelten Pollen wirken könnten, sind die gefundenen Konzentrationen an PA besorgniserregend. Es wird nicht davon ausgegangen, dass an Standorten, bei denen allein in 14 Tagesproben im Juli Konzentrationen von bis zu 48‘000 ng/g PA gefunden wurden, ein Verdünnungseffekt ausreicht, um Blütenpollen für den menschlichen Verzehr als unbedenklich einstufen zu können. Folglich sollten Pollenproben unbedingt hinsichtlich möglicher PA Konzentrationen untersucht werden, oder ferner das Sammeln von Pollen Ende Juni gestoppt werden, um die Qualität von Blütenpollen und Unbedenklichkeit hinsichtlich möglicher toxikologischer Risiken gewährleisten zu können.

5 Ausblick

Die vorliegende Arbeit konnte erste Einblicke in Untersuchungen hinsichtlich der Qualität von Blütenpollen in unterschiedlichen Regionen von Baden-Württemberg geben. Allerdings wurde anhand dieser Studien nur ein oberflächlicher Eindruck gewonnen.

In den hier dargestellten Studien wurde mit einer geringen Anzahl an Bienenvölkern gearbeitet, welche in weiteren Studien vergrößert werden muss, um aussagekräftige und stabile Ergebnisse erlangen zu können. Bislang konnte der tägliche Pestizideintrag in Pollenproben exemplarisch an einem intensiv bewirtschafteten Standort in einer Obstanbau-Region gezeigt werden. Informationen über den täglichen Pestizideintrag in anderen landwirtschaftlichen Regionen wie im Acker- oder Weinbau, oder auch in Naturregionen, konnten bislang nicht erlangt werden. Untersuchungen auch in diesen Regionen könnten zeigen, ob in allen landwirtschaftlichen Gebieten mit einem fast täglichen und hohen Eintrag an Pestizidkonzentrationen gerechnet werden muss, oder ob es andernfalls Zeitpunkte gibt, in denen durch Sammelpausen, Pestizidrückstände minimiert oder vermieden werden könnten.

Weitere Untersuchungen sollten ebenfalls hinsichtlich der Lagerfähigkeit von Blütenpollen durchgeführt werden. Aufgrund der geringen Anzahl an Proben muss die Aussagekraft der hier dargelegten Ergebnisse in weiteren Wiederholungen überprüft werden. Zudem konnten die Probensets der Jahre 2018 und 2019 aufgrund unterschiedlicher Methoden der Sequenzierungsfirmen nicht miteinander verglichen werden. Diese Erkenntnisse unterstreichen erneut, die Wichtigkeit der Vergleichbarkeit von Ergebnissen. Probensets sollten in folgenden Studien innerhalb eines Sequenzierungslabors analysiert werden. Unbeantwortet bleiben Fragen darüber, welche Veränderungen der MO-Zusammensetzungen sich unter Lagerung von weniger als sieben Tagen ergeben.

Analysen der PA Gehalte in den Proben zeigten erstmals exemplarisch, mit welchen PA Konzentrationen in Pollenproben aus dem Monat Juli in Baden-Württemberg zu rechnen ist. Informationen über PA Gehalte in anderen Monaten konnten nicht gewonnen werden. Folgende Studien sollten demnach Pollenproben aus allen Monaten von April bis August untersuchen, um geprüfte Aussagen darüber treffen zu können, in welchen Monaten mit erhöhten Risiken durch PAs in den Proben zu rechnen ist.

Standardisierte Untersuchungen von Blütenpollen können dazu beitragen, ein qualitativ hochwertiges Bienenprodukt als Nahrungsergänzung für den menschlichen Verzehr zu gewährleisten.

6 Zusammenfassung

6.1 Zusammenfassung

Honigbienen (*Apis mellifera*) sammeln zur Versorgung ihrer Brut neben Nektar auch Pollen von Pflanzen. Im Frühjahr sammeln Honigbienen Pollen von blühenden Pflanzen und Blumen und transportieren diese als sogenannte Pollenhöschen an ihren Hinterbeinen in den Bienenstock. Unter Zugabe von Nektar und Bienenspeichel wird der Pollen in Waben eingelagert, das Bienenbrot wird dann von Ammenbienen zur Herstellung von Larvenfuttersaft verwendet. Pollen liefern ein breites Spektrum an Nährstoffen, wie Proteine und Lipide, aber auch Kohlenhydrate, Vitamine und Enzyme. Aufgrund dieser Inhaltsstoffe ist Pollen auch für den Menschen attraktiv und wird als Nahrungsergänzungsmittel verwendet.

Honigbienen sammeln jedoch nicht nur Pollen von Wildpflanzen, sondern auch von blühenden Kulturen, die in der Landwirtschaft angebaut werden. Dementsprechend können Kontaminationen von Pflanzenschutzmitteln in Blütenpollen und Bienenbrot gefunden werden. Exemplarische Analysen können jedoch nur eine grobe Einschätzung über das Kontaminationsrisiko einer gesamten Sammelsaison geben.

Um einen tieferen Einblick über das Auftreten und die Verteilung von Pestizidrückständen während einer ganzen Saison zu erhalten, wurden von April bis Juli 2018 insgesamt 102 täglich gesammelte Pollenproben mit Hilfe von Pollenfallen in einem Obstanbaugebiet in Süddeutschland gesammelt (**Veröffentlichung 1**). Nahezu 90% der Pollenproben zeigten nachweisbare Konzentrationen an Pestizidrückständen. Insgesamt konnten 29 Pestizide, mit mehr als die Hälfte an Fungiziden, gefolgt von Insektiziden und Herbiziden in den Proben nachgewiesen werden. Maximale Konzentrationen von bis zu 4500 ng/g konnten Ende April gemessen werden. Auch Proben die Anfang Mai und Ende Juni gesammelt wurden, wiesen hohe Pestizidkonzentrationen auf. Ein allgemeines Risikomanagement wurde durchgeführt, um das Risiko der nachgewiesenen Pestizidkonzentrationen für Honigbienen abzuschätzen.

Die mikrobielle Qualität von Blütenpollen hängt stark von der botanischen und geografischen Herkunft, sowie den klimatischen Bedingungen und den Verarbeitungsschritten des Imkers nach der Ernte ab. Folgen nach der Ernte keine Verarbeitungsschritte wie Einfrieren oder Trocknung, kann das Wachstum von Mikroorganismen gefördert und die Pollenqualität durch negative Nebenwirkungen wie Fermentation oder Produktion von Mykotoxinen beeinflusst werden. Bakterien- und Pilzkolonien können sowohl durch kultivierungsabhängige Methoden wie Koloniezählung auf Platten als auch durch kultivierungsunabhängige Methoden wie der 16-rRNA-Amplikon Sequenzierung bestimmt werden.

Nach der Hypothese, dass Lagerbedingungen die Zusammensetzung von Mikroorganismen in Blütenpollen beeinflussen, wurde frisch geernteter Blütenpollen im Juni 2018 und 2019 sieben Tage lang unter definierten Bedingungen (kalt, Raumtemperatur, warm) gelagert und durch Sequenzierung von 16S- und 18S-PCR-Amplikons analysiert (**Veröffentlichung 2**). Die Bakteriengemeinschaft variierte geringfügig zwischen den untersuchten Standorten und zeigte keinen signifikanten Unterschied zwischen den Lagerbedingungen. Die Pilzgemeinschaft zeigte signifikante Unterschiede sowohl zwischen den untersuchten Standorten als auch zwischen den verschiedenen Lagerbedingungen. Die dominierenden Pilzgattungen in den Pollenproben waren *Cladosporium*, *Aspergillus* und *Zygosaccharomyces*. Während *Cladosporium* in frisch gesammeltem Pollen am dominantesten zu finden war und der prozentuale Anteil während der Lagerung abnahm, zeigten *Aspergillus* und *Zygosaccharomyces* eine signifikante Zunahme insbesondere unter warmen Lagerbedingungen. Die Zunahme dieser beiden Pilz- bzw. Hefegattungen könnte die Qualität von Pollen durch Fermentation oder Mykotoxinbildung negativ beeinflussen.

Auch andere, natürlich von Pflanzen produzierte Kontaminationen können negative Auswirkungen auf die menschliche Gesundheit haben. Pyrrolizidinalkaloide gehören zu einer Gruppe von sekundären Pflanzenstoffen, von denen mehr als 600 Strukturen in rund 3% aller blühenden

Pflanzen weltweit bekannt sind. PA sind bekannt, sowohl akute Vergiftungen als auch chronische Schädigungen oder Krebs bei Tieren und Menschen auslösen zu können. Lebensmittel botanischer Herkunft wie Tees, Kräuter, Honig und Blütenpollen können hohe PA-Werte aufweisen und wurden deshalb in mehreren Ländern intensiv untersucht.

Um das Wissen über PA-Kontaminationen in Blütenpollen zu erweitern und das Risiko der Konzentrationen abschätzen zu können, wurden im Juli 2019 an 57 Standorten in Baden-Württemberg Blütenpollen gesammelt und auf 42 verschiedene PA und deren N-Oxide untersucht (**Veröffentlichung 3**). Insgesamt wurden in über 90% aller untersuchten Proben 22 verschiedene PAs nachgewiesen. Nur 5% der PA wurden als PA von Pflanzen der *Senecio sp.* identifiziert, während 95% der PA mit botanischem Hintergrund von *Echium sp.* und *Eupatorium sp.* identifiziert werden konnten. Die maximale Konzentration der Summe an PA pro Probe wurde mit 48‘400 ng/g bestimmt, während der Median nur einen Wert von 44 ng/g Pollen aufwies. Dies resultierte aus der Tatsache, dass mehr als 60% der Proben Konzentrationen unter 500 ng/g aufwiesen. Nach den berechneten Risikowerten des BfR stellten jedoch immerhin 42% der Proben ein erhöhtes Risiko für die menschliche Gesundheit dar.

Die Studie zeigt deutlich, dass die Qualität von Blütenpollen durch verschiedene Faktoren wie Pestizidrückstände, mikrobielle Zusammensetzung und Pyrrolizidinalkaloide stark beeinflusst werden kann. Die Ergebnisse verdeutlichen die Wichtigkeit der Untersuchung verschiedener Einflussfaktoren sowie die Verwendung geeigneter Konservierungsmethoden, um ein Produkt von hoher Qualität zu erlangen.

6.2 Summary

Besides nectar, honey bees (*Apis mellifera*) are collecting pollen from plants to feeding their brood. From early spring on, pollen is collected by foraging honey bees from blooming crops and flowers and transported in pollen baskets known as corbicula via their legs into the hive. After adding nectar and secretion, pollen is stored in comb cells as bee bread and is used then by nurse bees to produce larval food. Pollen provides a wide spectrum of nutrients, as proteins and lipids, as well as carbohydrates, vitamins and enzymes. Due to these ingredients, bee pollen is also attractive for human and is used as food supplement in human nutrition.

Honey bees are not only collecting pollen from wild plants, but also from blooming crops and plants used in agricultural industry. Accordingly, bee bread and bee pollen were found to be frequently contaminated with pesticides. Pesticide residues in bee pollen can be monitored by using pollen traps installed in front of a hive which samples up to 40% of the daily amount from foraging bees, followed by a pesticide residues analysis. However, random analysis of pesticide residues can only provide an overview about the contamination risk over an entire season.

To get a deeper insight about the distribution and progression of pesticide contamination during an entire season, 102 daily bee pollen samples were collected by means of traps from April to July 2018 within a fruit-growing area in Southern Germany (**publication 1**). Almost 90% of the pollen samples showed detectable concentrations of pesticide residues. In total 29 pesticides were detected with half of them as fungicides, followed by insecticides and herbicides. Maximum concentrations measured up to 4500 ng/g pollen in April, while also in first half of May and second half of June high amounts of residues could be detected. A general risk management was proposed to estimate the risk of the detected pesticides concentrations to honey bees.

The microbiological quality of bee pollen strongly depends on the botanical and geographical origin, the climatic conditions and on post-harvest processing steps by the bee keeper. When

no processing like freezing or drying is followed after harvesting, growth of microorganisms can influence the pollen quality by negative side effects as fermentation or mycotoxin production. Bacterial and fungal colonies can be examined by cultivation-dependent methods, e.g. colony counts on plates, or by cultivation-independent methods such as 16-rRNA amplicon sequencing.

Due to the hypothesis, that storage conditions influence the composition of microorganisms, freshly harvested bee pollen from June 2018 and 2019 was stored for seven days under defined cold to warm conditions and analyzed by next-generation sequencing of 16S and 18S PCR-amplicons (**publication 2**). Bacterial communities varied slightly between different locations and showed no significant difference between storage conditions, whereas the fungal composition showed significant effects between location and storage conditions. Most dominant fungal species in pollen were *Cladosporium, Aspergillus* and *Zygosaccharomyces*. While the fungus *Cladosporium* was present in fresh collected bee pollen and decreased during storage, *Aspergillus* and *Zygosaccharomyces* proportion increased, especially under warm storage conditions. The increase of these two fungus genera could negatively influence the pollen quality by fermentation or mycotoxin production if it is used as a dietary supplement.

Also, other natural contaminates produced by plants can have negative effects on human health. Pyrrolizidine alkaloids are a group of secondary plant defense compounds, which are known for more than 600 structures in around 3% of all flowing plants worldwide. PAs are known as hepatotoxins for animals and humans, as they can show acute poisoning or possible causing cancer. Foods with botanical origin like teas, herbals or honey and bee pollen can show significant values of PAs and were studies from several countries worldwide.

To increase the knowledge of PA contamination and estimate the risk in bee collected pollen from Germany, bee pollen was collected seasonal in July 2019 at 57 locations and analyzed for 42 different PAs and their N-oxides (**publication 3**). In total, 22 different PAs were detected in

over 90% of all analyzed samples. Only 5% of the PAs were identified as PAs from *Senecio sp.,* while 95% of the PAs could be identified with botanical background from *Echium sp.* and *Eupatorium sp.* Maximum ΣPA measured up to 48’400 ng/g pollen, while the median of ΣPA showed only a value of 44 ng/g pollen. This was due to the fact, that more than 60% of the samples featured values below 500 ng/g pollen. According to the calculated risk values of BfR, 42% of the samples represented an increased risk for human health.

The study clearly demonstrates that the quality of bee pollen can be strongly influenced by several factors, such as pesticide residues, microbial composition, and pyrrolizidine alkaloid compounds. The results illustrate the importance of investigating various influencing factors and the use of suitable preservation methods to obtain a product of high quality.

7 Literaturverzeichnis

Aktar, M. W.; Sengupta, sD.; Chowdhury, A. (2009): Impact of pesticides use in agriculture: their benefits and hazards. In: *Interdisciplinary Toxicology* 2 (1), S. 1–12. DOI: 10.2478/v10102-009-0001-7.

Álvarez-Ruiz, R.; Picó, Y.; Sadutto, D.; Campo, J. (2021): Development of multi-residue extraction procedures using QuEChERS and liquid chromatography tandem mass spectrometry for the determination of different types of organic pollutants in mussel. In: *Analytical and bioanalytical chemistry* 413 (15), S. 4063–4076. DOI: 10.1007/s00216-021-03363-y.

Anastassiades, M.; Lehotay, S. J.; Štajnbaher, D.; Schenck, Frank J. (2003): Fast and Easy Multiresidue Method Employing Acetonitrile Extraction/Partitioning and "Dispersive Solid-Phase Extraction" for the Determination of Pesticide Residues in Produce. In: *J AOAC Int.* 86 (2), S. 412–431. DOI: 10.1093/jaoac/86.2.412.

Anderson, K. E.; Carroll, M. J.; Sheehan, T.; Lanan, M. C.; Mott, B. M.; Maes, P. Corby-Harris, V. (2014a): Hive-stored pollen of honey bees: many lines of evidence are consistent with pollen preservation, not nutrient conversion. In: *Molecular ecology* 23 (23), S. 5904–5917. DOI: 10.1111/mec.12966.

Anderson, K. E.; Carroll, M. J.; Sheehan, T.; Lanan, M. C.; Mott, B. M.; Maes, P.; Corby-Harris, V. (2014b): Hive-stored pollen of honey bees: many lines of evidence are consistent with pollen preservation, not nutrient conversion. In: *Molecular ecology* 23 (23), S. 5904–5917. DOI: 10.1111/mec.12966.

Aupinel, P.; Fortini, D.; Michaud, B.; Marolleau, F.; Tasei, J.-N.; Odoux, J.-F. (2007): Toxicity of dimethoate and fenoxycarb to honey bee brood (Apis mellifera), using a new in vitro standardized feeding method. In: *Pest management science* 63 (11), S. 1090–1094. DOI: 10.1002/ps.1446.

Avni, D.; Hendriksma, H. P.; Dag, A.; Uni, Z.; Shafir, S. (2014): Nutritional aspects of honey bee-collected pollen and constraints on colony development in the eastern Mediterranean. In: *Journal of insect physiology* 69, S. 65–73. DOI: 10.1016/j.jinsphys.2014.07.001.

Baiswar, P.; Chandra, S.; Kumar, R.; Ngachan, S. V.; Munda, G. C. (2010): First report of powdery mildew caused by Podosphaera sp. on Hibiscus sabdariffa in India. In: *Austral. Plant Disease Notes* 5 (1), S. 123. DOI: 10.1071/DN10045.

Balinova, A. M.; Mladenova, R. I.; Shtereva, D. D. (2006): Effects of processing on pesticide residues in peaches intended for baby food. In: *Food additives and contaminants* 23 (9), S. 895–901. DOI: 10.1080/02652030600771715.

Bárbara, M.; Machado, C.; Sodré, G.; Dias, L.; Estevinho, L.; Carvalho, C. de (2015): Microbiological Assessment, Nutritional Characterization and Phenolic Compounds of Bee Pollen from Mellipona mandacaia Smith, 1983. In: *Molecules* 20 (7), S. 12525–12544. DOI: 10.3390/molecules200712525.

Bartomeus, I.; Potts, Simon G.; Steffan-Dewenter, I.; Vaissière, B. E.; Woyciechowski, M.; Krewenka, K. M. et al. (2014): Contribution of insect pollinators to crop yield and quality varies with agricultural intensification. In: *PeerJ* 2, e328. DOI: 10.7717/peerj.328.

Basim, E.; Basim, H.; Özcan, M. (2006): Antibacterial activities of Turkish pollen and propolis extracts against plant bacterial pathogens. In: *Journal of Food Engineering* 77 (4), S. 992–996. DOI: 10.1016/j.jfoodeng.2005.08.027.

Beekman, M.; Ratnieks, F. L. W. (2000): Long-range foraging by the honey-bee, Apis mellifera L. In: *Funct Ecology* 14 (4), S. 490–496. DOI: 10.1046/j.1365-2435.2000.00443.x.

Beliën, T.; Kellers, J.; Heylen, K.; Keulemans, W.; Billen, J.; Arckens, L. et al. (2009): Effects of sublethal doses of crop protection agents on honey bee (Apis mellifera) global colony vitality and its potential link with aberrant foraging activity. In: *Communications in agricultural and applied biological sciences* 74 (1), S. 245–253.

Betteridge, K; Cao, Y.; Colegate, S. M. (2005): Improved method for extraction and LC-MS analysis of pyrrolizidine alkaloids and their N-oxides in honey: application to Echium vulgare honeys. In: *J. Agric. Food Chem.* 53 (6), S. 1894–1902. DOI: 10.1021/jf0480952.

BfR (2013): Pyrrolizidine alkaloids in herbal teas and teas - BfR opinion 018/2013 of July 5, 2013. Pyrrolizidinalkaloide in Kräutertees und Tees - Stellungnahme 018/2013 des BfR vom 5. Juli 2013. Online verfügbar unter https://pubs.acs.org/doi/pdf/10.1021/np50014a001?casa_token=i_jyVfgj0PgAAAAA:D7bEQf0pyUjJyiN-hCgLEfPTo3Z0KzJR3MuD_a3vNkwNSXsJNAXvmbkZdY9jSiPFIwjrhNkQn3JYfFYb6, zuletzt geprüft am 22.01.2021.

BfR (2020): Updated risk assessment on the content of 1,2-unsaturated pyrrolizidine alkaloids (PA) in food: BfR Opinion No. 026/2020 of June 17, 2020. Updated risk assessment on the content of 1,2-unsaturated pyrrolizidine alkaloids (PA) in food: BfR Opinion No. 026/2020 of June 17, 2020 Aktualisierte Risikobewertung zu Gehalten an 1,2-ungesättigten Pyrrolizidinalkaloiden (PA) in Lebensmitteln: Stellungnahme Nr. 026/2020 des BfR vom 17. Juni 2020. Unter Mitarbeit von German Federal Institute for Risk Management. Online verfügbar unter https://www.bfr.bund.de/cm/343/aktualisierte-risikobewertung-zu-gehalten-an-1-2-ungesaettigten-pyrrolizidinalkaloiden-pa-in-lebensmitteln.pdf, zuletzt geprüft am 22.01.2021.

BienSchV (1992): Verordnung über die Anwendung bienengefährlicher Pflanzenschutzmittel. Bienenschutzverordnung. Fundstelle: Bundesministerium der Justiz und für Verbraucherschutz, Bundesamt für Justiz. Online verfügbar unter http://www.gesetze-im-internet.de/bienschv_1992/BienSchV_1992.pdf.

BMEL (2021): Ökologischer Landbau in Deutschland. Online verfügbar unter https://www.bmel.de/SharedDocs/Downloads/DE/Broschueren/OekolandbauDeutschland.pdf?__blob=publicationFile&v=9, zuletzt geprüft am 10.03.2021.

Bodi, D.; Ronczka, S.; Gottschalk, C.; Behr, N.; Skibba, A.; Wagner, M. et al. (2014): Determination of pyrrolizidine alkaloids in tea, herbal drugs and honey. In: *Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment* 31 (11), S. 1886–1895. DOI: 10.1080/19440049.2014.964337.

Böhme, F.; Bischoff, G.; Zebitz, C. P. W.; Rosenkranz, P.; Wallner, K. (2018): Pesticide residue survey of pollen loads collected by honeybees (Apis mellifera) in daily intervals at three agricultural sites in South Germany. In: *PloS one* 13 (7), e0199995. DOI: 10.1371/journal.pone.0199995.

Bonvehí, J. S.; Jordà, R. E. (1997): Nutrient Composition and Microbiological Quality of Honeybee-Collected Pollen in Spain. In: *J. Agric. Food Chem.* (45), S. 725–732, zuletzt geprüft am 21.04.2020.

Boppré, M. l.; Colegate, S. M.; Edgar, J. A. (2005): Pyrrolizidine alkaloids of Echium vulgare honey found in pure pollen. In: *Journal of agricultural and food chemistry* 53 (3), S. 594–600. DOI: 10.1021/jf0484531.

Botías, C.; David, A.; Hill, E. M.; Goulson, D. (2016): Contamination of wild plants near neonicotinoid seed-treated crops, and implications for non-target insects. In: *The Science of the total environment* 566-567, S. 269–278. DOI: 10.1016/j.scitotenv.2016.05.065.

Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (2020): Online Datenbank von zugelassenen Pflanzenschutzmitteln. Online verfügbar unter https://apps2.bvl.bund.de/psm/jsp/index.jsp, zuletzt geprüft am April 2020.

BVL (2021a): Fleisch richtig lagern und verarbeiten. Hg. v. Bundesamt für Verbraucherschutz ung Lebensmittelsicherheit. Online verfügbar unter https://www.bvl.bund.de/DE/Arbeitsbereiche/01_Lebensmittel/03_Verbraucher/03_UmgangLM/02_LMzubereiten/01_Fleisch/lm_zubereitung_fleisch_basepage.html, zuletzt aktualisiert am 12.03.2021, zuletzt geprüft am 12.03.2021.

BVL (2021b): Lister zugelassener Pflanzenschutzmittel in Deutschland mit Informationen über beendete Zulassungen. Stand Januar 2021. Hg. v. Bundesamt für Verbraucherschutz und Lebensmittelsicherheit. Online verfügbar unter www.bvl.bund.de/infopsm.

BVL (2021c): Überblick über lebensmittelbedingte Infektionen und Intoxikationen. Hg. v. Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, zuletzt aktualisiert am 12.03.2021, zuletzt geprüft am 12.03.2021.

Campos, M. G.; Cunha, A.; Markham, K. R. (1997): Bee-Pollen 5, S. 93–100. DOI: 10.1007/978-1-4757-9371-0_12.

Campos, M. G. R.; Frigerio, C.; Lopes, J.; Bogdanov, S. (2010): What is the future of Bee-Pollen? In: *J. of ApiProd ApiMed Sci* 2 (4), S. 131–144. DOI: 10.3896/IBRA.4.02.4.01.

Cardinal, S.; Danforth, B. N. (2013): Bees diversified in the age of eudicots. In: *Proceedings of the Royal Society.* 280: 20122686. http://dx.doi.org/10.1098/rspb.2012.2686.

Carpenter, K. J. (2003): A short history of nutritional science: part 4 (1945-1985). In: *The Journal of nutrition* 133 (11), S. 3331–3342. DOI: 10.1093/jn/133.11.3331.

Chan, P.; Hasemann, J. K.; Prejean, J. D.; Nyska, A. (2003): Toxicity and carcinogenicity of riddelliine in rats and mice. In: *Toxicology letters* 144 (3), S. 295–311. DOI: 10.1016/S0378-4274(03)00240-6.

Chauzat, M.-P; Faucon, J.-P.; Martel, A.-C.; Lachaize, J.; Cougoule, N.; Aubert, M. (2006): A survey of pesticide residues in pollen loads collected by honey bees in France. In: *Journal of economic entomology* 99 (2), S. 253–262. DOI: 10.1603/0022-0493-99.2.253.

Cobert, S. A.; Fussel, M.; AKE, R.; FRASER, A.; GUNSON, C.; SAVAGE, A.; SMITH, K. (1993): Temperature and the pollinating activity of social bees. In: *Ecol Entomol* 18 (1), S. 17–30. DOI: 10.1111/j.1365-2311.1993.tb01075.x.

Codling, G.; Al Naggar, Y.; Giesy, J. P.; Robertson, A. J. (2016): Concentrations of neonicotinoid insecticides in honey, pollen and honey bees (Apis mellifera L.) in central Saskatchewan, Canada. In: *Chemosphere* 144, S. 2321–2328. DOI: 10.1016/j.chemosphere.2015.10.135.

Codling, G.; Naggar, Y. A.; Giesy, J. P.; Robertson, A. J. (2018): Neonicotinoid insecticides in pollen, honey and adult bees in colonies of the European honey bee (Apis mellifera L.) in Egypt. In: *Ecotoxicology (London, England)* 27 (2), S. 122–131. DOI: 10.1007/s10646-017-1876-2.

Colegate, S. M.; Stegelmeier, B. L.; Edgar, J. A. (2012): Dietary exposure of livestock and humans to hepatotoxic natural products. In: Johanna Fink-Gremmels (Hg.): Animal feed contamination. Effects on livestock and food safety. Cambridge, UK, Philadelphia: Woodhead Publishing Limited (Woodhead Publishing series in food science, technology and nutrition, no. 215), S. 352–382. Online verfügbar unter https://link.springer.com/content/pdf/10.1007/s11356-021-13932-4.pdf.

Corby-Harris, V.; Maes, P.; Anderson, K. E. (2014): The bacterial communities associated with honey bee (Apis mellifera) foragers. In: *PloS one* 9 (4), e95056. DOI: 10.1371/journal.pone.0095056.

Craddock, H. A.; Huang, D.; Turner, P. C.; Quirós-Alcalá, L.; Payne-Sturges, D. C. (2019): Trends in neonicotinoid pesticide residues in food and water in the United States, 1999-2015. In: *Environmental health : a global access science source* 18 (1), S. 7. DOI: 10.1186/s12940-018-0441-7.

Crailsheim, K.; Schneider, L.H.W.; Hrassnigg, N.; Bühlmann, G.; Brosch, U.; Gmeinbauer, R.; Schöffmann, B. (1992): Pollen consumption and utilization in worker honeybees (Apis mellifera carnica): Dependence on individual age and function. In: *Journal of insect physiology* 38 (6), S. 409–419. DOI: 10.1016/0022-1910(92)90117-V.

Crous, P. W.; Wingfield, M. J.; Mansilla, J. P.; Alfenas, A. C.; Groenewald, J. Z. (2006): Phylogenetic reassessment of Mycosphaerella spp. and their anamorphs occurring on Eucalyptus. II. In: *Studies in mycology* 55, S. 99–131. DOI: 10.3114/sim.55.1.99.

Cruden, R. W. (1999): Pollen grains: why so many? In: *Plant Systematics and Evolution* (222), S. 143–165.

Czoppelt, C. (1991): Toxicity measurements with the juvenoid fenoxycarb on bee larvae in the in vitro breeding test. (Toxizitätsmessungen mit dem Juvenoid Fenoxycarb an Bienenlarven im in vitro Aufzuchttest). In: *Apidologie* (22 (4)), S. 457–459, zuletzt geprüft am 24.06.2020.

D'Alvise, P.; Böhme, F.; Codrea, M. C.; Seitz, Alexander; N., Sven; Binzer, M. et al. (2018): The impact of winter feed type on intestinal microbiota and parasites in honey bees. In: *Apidologie* 49 (2), S. 252–264. DOI: 10.1007/s13592-017-0551-1.

Darwin, C. (1859): On the Origin of Species by Means of Natural Selection; Or, The Preservation of Favoured Races in the Struggle for Life. Online verfügbar unter http://darwin-online.org.uk/converted/pdf/1861_OriginNY_F382.pdf, zuletzt geprüft am 08.03.2021.

David, A.; Botías, C.; Abdul-Sada, A.; Goulson, D.; Hill, E. M. (2015): Sensitive determination of mixtures of neonicotinoid and fungicide residues in pollen and single bumblebees using a scaled down QuEChERS method for exposure assessment. In: *Analytical and bioanalytical chemistry* 407 (26), S. 8151–8162. DOI: 10.1007/s00216-015-8986-6.

David, A.; Botías, C.; Abdul-Sada, A.; Nicholls, E.; Rotheray, E. L.; Hill, E.h M.; Goulson, D. (2016): Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops. In: *Environment international* 88, S. 169–178. DOI: 10.1016/j.envint.2015.12.011.

DeBiMo (2017-2020): Deutsches Bienenmonitoring. Online verfügbar unter https://bienenmonitoring.uni-hohenheim.de/fileadmin/einrichtungen/bienenmonitoring/Abschlussbericht_DeBiMo_2017-2019.pdf.

De-Melo, A. A. M.; Estevinho, M. L. M. F.; Almeida-Muradian, L. B. (2015): A diagnosis of the microbiological quality of dehydrated bee-pollen produced in Brazil. In: *Letters in applied microbiology* 61 (5), S. 477–483. DOI: 10.1111/lam.12480.

De-Melo, A. A. M.; Estevinho, M. L. M. F.; Sattler, J. A. G.; Souza, B. R.; Freitas, A. da Silva; Barth, O. M.; Almeida-Muradian, L. B. (2016): Effect of processing conditions on characteristics of dehydrated bee-pollen and correlation between quality parameters. In: *LWT - Food Science and Technology* 65, S. 808–815. DOI: 10.1016/j.lwt.2015.09.014.

Detroy, B. F.; Harp, E. R.; United States. Agricultural Research Service; University of Wisconsin. College of Agricultural and Life Sciences (1976): Trapping pollen from honey bee colonies: U.S. Department of Agriculture, Agricultural Research Service (Production research report). Online verfügbar unter https://books.google.de/books?id=iCkic0NL6zoC.

Deutsches Bienenmonitoring (2014-2019). Hg. v. University of Hohenheim. Online verfügbar unter https://bienenmonitoring.uni-hohenheim.de/, zuletzt geprüft am 22.06.2020.

Dinkov, D. (2016): Microorganisms in Vacuum Stored Flower Bee Pollen. In: *J Bacteriol Virol* 46 (4), S. 258. DOI: 10.4167/jbv.2016.46.4.258.

Dinkov, D. (2018): Isolation and Identification of Bacteria in Flower Bee Pollen. In: *Walailak Journal Sci & Tech* (15 (3)), S. 225–234, zuletzt geprüft am 20.04.2020.

Disayathanoowat, T.; Li, H. Y.; Supapimon, N.; Suwannarach, N.; Lumyong, S.; Chantawannakul, P.; Guo, J. (2020): Different Dynamics of Bacterial and Fungal Communities in Hive-Stored Bee Bread and Their Possible Roles: A Case Study from Two Commercial Honey Bees in China. In: *Microorganisms* 8 (2). DOI: 10.3390/microorganisms8020264.

Donkersley, P.; Rhodes, G.; Pickup, R. W.; Jones, K. C.; Wilson, K. (2018): Bacterial communities associated with honeybee food stores are correlated with land use. In: *Ecology and evolution* 8 (10), S. 4743–4756. DOI: 10.1002/ece3.3999.

Drummond, F. A.; Ballman, E. S.; Eitzer, B. D.; Du Clos, B.; Dill, J. (2018): Exposure of Honey Bee (Apis mellifera L.) Colonies to Pesticides in Pollen, A Statewide Assessment in Maine. In: *Environmental entomology* 47 (2), S. 378–387. DOI: 10.1093/ee/nvy023.

Dübecke, A.; Beckh, G.; Lüllmann, C. (2011): Pyrrolizidine alkaloids in honey and bee pollen. In: *Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment* 28 (3), S. 348–358. DOI: 10.1080/19440049.2010.541594.

DWD (2018): Deutschlandwetter im Jahr 2018. Unter Mitarbeit von Deutscher Wetterdienst. Online verfügbar unter https://www.dwd.de/DE/presse/pressemitteilungen/DE/2018/20181228_deutschlandwetter_jahr2018.pdf?__blob=publicationFile&v=3.

DWD (2019): Deutschlandwetter im Jahr 2019. Unter Mitarbeit von Deutscher Wetterdienst. Online verfügbar unter https://www.dwd.de/DE/presse/pressemitteilungen/DE/2019/20191230_deutschlandwetter_jahr2019.pdf?__blob=publicationFile&v=3.

DWD (2020): Deutschlandwetter im Jahr 2020. Unter Mitarbeit von Deutscher Wetterdienst. Online verfügbar unter https://www.dwd.de/DE/presse/pressemitteilungen/DE/2020/20201230_deutschlandwetter_jahr_2020.pdf?__blob=publicationFile&v=3.

Edgar, J. A.; Colegate, S. M.; Boppré, M.; Molyneux, R. J. (2011): Pyrrolizidine alkaloids in food: a spectrum of potential health consequences. In: *Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment* 28 (3), S. 308–324. DOI: 10.1080/19440049.2010.547520.

EFSA (2010): Modification of the existing MRLs for tebuconazole in various citrus fruits. In: *EFSA Journal* 8 (11), S. 1896. DOI: 10.2903/j.efsa.2010.1896.

EFSA (2011): Scientific Opinion on Pyrrolizidine alkaloids in food and feed (EFSA-Q-2010-01004). DOI: 10.2903/j.efsa.2011.2406.

EFSA (2017): Risks for human health related to the presence of pyrrolizidine alkaloids in honey, tea, herbal infusions and food supplements. In: *EFSA journal. European Food Safety Authority* 15 (7), e04908. DOI: 10.2903/j.efsa.2017.4908.

EFSA; Cabrera, L. C.; Pastor, P. M. (2021): THe 2019 European Union Report on Pesticide Residues in Food. National summary reports on pesticide residue analysis performed in 2019. In: *EFS3* 18 (4). DOI: 10.2903/sp.efsa.2021.EN-6487.

EPA (2007): Pesticide Industry Sales and Usage Report: 2006 and 2007 Market Estimates. Online verfügbar unter http://www.panna.org/sites/default/files/EPA%20market_estimates2007.pdf, zuletzt geprüft am 10.03.2021.

Estevinho, L. M.; Rodrigues, S.; Pereira, A. P.; Feás, X. (2012): Portuguese bee pollen: palynological study, nutritional and microbiological evaluation. In: *International Journal of Food Science & Technology* 47 (2), S. 429–435. DOI: 10.1111/j.1365-2621.2011.02859.x.

Fatrcová-Šramková, K.; Nôžková, J.; Máriássyová, M.; Kačániová, M. (2016): Biologically active antimicrobial and antioxidant substances in the Helianthus annuus L. bee pollen. In: *Journal of environmental science and health. Part. B, Pesticides, food contaminants, and agricultural wastes* 51 (3), S. 176–181. DOI: 10.1080/03601234.2015.1108811.

Favaro, R.; Bauer, L. M.; Rossi, M.; D'Ambrosio, L.; Bucher, E.; Angeli, S. (2019): Botanical Origin of Pesticide Residues in Pollen Loads Collected by Honeybees During and After Apple Bloom. In: *Frontiers in physiology* 10, S. 1069. DOI: 10.3389/fphys.2019.01069.

Feás, X.; Vázquez-Tato, M. P.; Estevinho, L.; Seijas, J. A.; Iglesias, A. (2012): Organic bee pollen: botanical origin, nutritional value, bioactive compounds, antioxidant activity and microbiological quality. In: *Molecules (Basel, Switzerland)* 17 (7), S. 8359–8377. DOI: 10.3390/molecules17078359.

Free, J. B. (1963): The Flower Constancy of Honeybees. In: *The Journal of Animal Ecology* 32 (1), S. 119–131. DOI: 10.2307/2521.

Fridman, S.; Izhaki, I.; Gerchman, Y.; Halpern, M. (2012): Bacterial communities in floral nectar. In: *Environmental microbiology reports* 4 (1), S. 97–104. DOI: 10.1111/j.1758-2229.2011.00309.x.

Friedle, C.; D'Alvise, P.; Schweikert, K.; Wallner, K.; Hasselmann, M. (2021a): Changes of microorganism composition in fresh and stored bee pollen from Southern Germany. In: *Environ Sci Pollut Res*. DOI: 10.1007/s11356-021-13932-4.

Friedle, C.; Wallner, K.; Rosenkranz, P.; Martens, D.; Vetter, W. (2021b): Pesticide residues in daily bee pollen samples (April–July) from an intensive agricultural region in Southern Germany. In: *Environ Sci Pollut Res*, S. 1–15. DOI: 10.1007/s11356-020-12318-2.

GBE (2017): Average body size of the population (height in m, weight in kg). Structural characteristics: years, Germany, age, gender. Durchschnittliche Körpermaße der Bevölkerung (Größe in m, Gewicht in kg). Gliederungsmerkmale: Jahre, Deutschland, Alter, Geschlecht. Hg. v. Federal Health Reporting Germany. Online verfügbar unter https://www.gbe-bund.de/gbe/pkg_isgbe5.prc_menu_olap?p_uid=gast&p_aid=54890293&p_sprache=D&p_help=3&p_indnr=223&p_indsp=&p_ityp=H&p_fid=.

Gill, R. J.; Ramos-Rodriguez, O.; Raine, N. E. (2012): Combined pesticide exposure severely affects individual- and colony-level traits in bees. In: *Nature* 491 (7422), S. 105–108. DOI: 10.1038/nature11585.

González, G.; Hinojo, M. J.; Mateo, R.; Medina, A.; Jiménez, M. (2005): Occurrence of mycotoxin producing fungi in bee pollen. In: *International journal of food microbiology* 105 (1), S. 1–9. DOI: 10.1016/j.ijfoodmicro.2005.05.001.

Gottschalk, C.; Huckauf, A.; Dübecke, A.; Kaltner, F.; Zimmermann, M.; Rahaus, I.; Beuerle, T. (2018): Uncertainties in the determination of pyrrolizidine alkaloid levels in naturally contaminated honeys and comparison of results obtained by different analytical approaches. In: *Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment* 35 (7), S. 1366–1383. DOI: 10.1080/19440049.2018.1468929.

Halpern, M.; Fridman, S.; Atamna-Ismaeel, N.; Izhaki, I. (2013): Rosenbergiella nectarea gen. nov., sp. nov., in the family Enterobacteriaceae, isolated from floral nectar. In: *International journal of systematic and evolutionary microbiology* 63 (Pt 11), S. 4259–4265. DOI: 10.1099/ijs.0.052217-0.

Hamilton, D.; Ambrus, A.; Dieterle, R.; Felsot, A.; Harris, C.; Petersen, B. et al. (2004): Pesticide residues in food--acute dietary exposure. In: *Pest management science* 60 (4), S. 311–339. DOI: 10.1002/ps.865.

Hani, B.; Dalila, B.; Saliha, D.; Daoud, H.; Mouloud, G.; Seddik, K. (2012): Microbioligical Sanitary Aspects of Pollen. In: *Advances in Environmental Biology* (6), S. 1415–1420, zuletzt geprüft am 21.07.2020.

Hartmann, T.; Toppel, G. (1987): Senecionine n-oxide, the primary product of pyrrolizidine alkaloid biosynthesis in root cultures of Senecio vulgaris. In: *Phytochemistry* 26 (6), S. 1639–1643. DOI: 10.1016/S0031-9422(00)82261-X.

Harz M. (2014): Jahreslauf - Phänologischer Bienenweidekalender. Hg. v. Die Honigmacher (Harz M. und Bienenweide Apis e.V.). Online verfügbar unter https://www.die-honigmacher.de/kurs2/kapitel_20000.html.

Haydak, M. H. (1970): Honey Bee Nutrition. In: *Annu. Rev. Entomol.* 15 (1), S. 143–156. DOI: 10.1146/annurev.en.15.010170.001043.

Hung, K.-L. J.; Kingston, J. M.; Albrecht, M.; Holway, D. A.; Kohn, J. R. (2018): The worldwide importance of honey bees as pollinators in natural habitats. In: *Proceedings. Biological sciences* 285 (1870). DOI: 10.1098/rspb.2017.2140.

Kakar, F.; Akbarian, Z.; Leslie, T.; Mustafa, M. L.; Watson, J.; van Egmond, H. P. et al. (2010): An outbreak of hepatic veno-occlusive disease in Western afghanistan associated with exposure to wheat flour contaminated with pyrrolizidine alkaloids. In: *Journal of toxicology* 2010, S. 313280. DOI: 10.1155/2010/313280.

Kaltner, F.; Rychlik, M.; Gareis, M.; Gottschalk, C. (2018): Influence of Storage on the Stability of Toxic Pyrrolizidine Alkaloids and Their N-Oxides in Peppermint Tea, Hay, and Honey. In: *Journal of agricultural and food chemistry* 66 (20), S. 5221–5228. DOI: 10.1021/acs.jafc.7b06036.

Kaltner, F.; Rychlik, M.; Gareis, M.; Gottschalk, C. (2020): Occurrence and Risk Assessment of Pyrrolizidine Alkaloids in Spices and Culinary Herbs from Various Geographical Origins. In: *Toxins* 12 (3), S. 155. DOI: 10.3390/toxins12030155.

Kamel, A. (2010): Refined methodology for the determination of neonicotinoid pesticides and their metabolites in honey bees and bee products by liquid chromatography-tandem mass spectrometry (LC-MS/MS). In: *Journal of agricultural and food chemistry* 58 (10), S. 5926–5931. DOI: 10.1021/jf904120n.

Kast, C.; Kilchenmann, V.; Reinhard, H.; Bieri, K.; Zoller, O. (2019): Pyrrolizidine Alkaloids: The Botanical Origin of Pollen Collected during the Flowering Period of Echium vulgare and the Stability of Pyrrolizidine Alkaloids in Bee Bread. In: *Molecules (Basel, Switzerland)* 24 (12). DOI: 10.3390/molecules24122214.

Kast, C.; Kilchenmann, V.; Reinhard, H.; Droz, B.; Lucchetti, M. A.; Dübecke, A. et al. (2018): Chemical fingerprinting identifies Echium vulgare, Eupatorium cannabinum and Senecio spp. as plant species mainly responsible for pyrrolizidine alkaloids in bee-collected pollen. In: *Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment* 35 (2), S. 316–327. DOI: 10.1080/19440049.2017.1378443.

Keller, I.; Fluri, P.; Imdorf, A. (2005): Pollen nutrition and colony development in honey bees—Part II. In: *Bee World* 86 (2), S. 27–34. DOI: 10.1080/0005772X.2005.11099650.

Kempf, M.; Beuerle, T.; Bühringer, M.; Denner, M.; Trost, D.; Ohe, K. von der et al. (2008): Pyrrolizidine alkaloids in honey: risk analysis by gas chromatography-mass spectrometry. In: *Molecular nutrition & food research* 52 (10), S. 1193–1200. DOI: 10.1002/mnfr.200800051.

Kempf, M.; Heil, S.; Hasslauer, I.; Schmidt, L.; Ohe, K. von der; Theuring, C. et al. (2010a): Pyrrolizidine alkaloids in pollen and pollen products. In: *Molecular nutrition & food research* 54 (2), S. 292–300. DOI: 10.1002/mnfr.200900289.

Kempf, M.; Reinhard, A.; Beuerle, T. (2010b): Pyrrolizidine alkaloids (PAs) in honey and pollen-legal regulation of PA levels in food and animal feed required. In: *Molecular nutrition & food research* 54 (1), S. 158–168. DOI: 10.1002/mnfr.200900529.

Kempf, M.; Wittig, M.; Schönfeld, K.; Cramer, L.; Schreier, P.; Beuerle, T. (2011): Pyrrolizidine alkaloids in food: downstream contamination in the food chain caused by honey and pollen. In: *Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment* 28 (3), S. 325–331. DOI: 10.1080/19440049.2010.521771.

Kevan, P. G.; Baker, H. G. (1983): Insects as Flower Visitors and Pollinators. In: *Annu. Rev. Entomol.* 28 (1), S. 407–453. DOI: 10.1146/annurev.en.28.010183.002203.

Keweloh, H. (2016): Mikroorganismen in Lebensmitteln. Theorie und Praxis der Lebensmittelhygiene. 6. Auflage. Haan-Gruiten: Pfanneberg. Online verfügbar unter http://www.nicra-icar.in/nicrarevised/images/publications/Research_art/HRT20_Potential%20impact%20of%20climate%20change.pdf, zuletzt geprüft am 12.03.2021.

Klein, A.-M.; Vaissière, B. E.; Cane, J. H.; Steffan-Dewenter, I.; Cunningham, S. A.; Kremen, C.; Tscharntke, T. (2007): Importance of pollinators in changing landscapes for world crops. In: *Proceedings. Biological sciences* 274 (1608), S. 303–313. DOI: 10.1098/rspb.2006.3721.

Klumpp, S. (2013): Palynologie - grundlagen, arbeitsmethoden und auswertung. [Place of publication not identified]: Grin Verlag.

Koch, H.; Weißer, P. (1997): Exposure of honey bees during pesticide application under field conditions. In: *Apidologie* 28 (6), S. 439–447. DOI: 10.1051/apido:19970610.

Koesukwiwat, U.; Sanguankaew, K.; Leepipatpiboon, N. (2014): Evaluation of a modified QuEChERS method for analysis of mycotoxins in rice. In: *Food chemistry* 153, S. 44–51. DOI: 10.1016/j.foodchem.2013.12.029.

Kostić, A.Ž.; Barać, M.B.; Stanojević, S. P.; Milojković-Opsenica, D. M.; Tešić, Ž.Lj; Šikoparija, B. (2015): Physicochemical composition and techno-functional properties of bee pollen collected in Serbia. In: *LWT - Food Science and Technology* 62 (1), S. 301–309. DOI: 10.1016/j.lwt.2015.01.031.

Kwong, W. K.; Moran, N. A. (2016): Gut microbial communities of social bees. In: *Nature reviews. Microbiology* 14 (6), S. 374–384. DOI: 10.1038/nrmicro.2016.43.

Ladányi, M.; Persely, Sz.; Szabó, T.; Szabó, Z.; Soltész, M.; Nyéki, J. (2010): Climatic indicator analysis of blooming time for sour cherries. In: *International Journal of Horticultural Science* (16), S. 11–16. Online verfügbar unter https://core.ac.uk/reader/161872307.

Lawrence, T. (2015): Pollination and Protecting Bees and Other Pollinators. Home Garden Series. In: *Washington State University Extension*. DOI: 10.13140/RG.2.1.3099.1203.

Lehotay, S. J.; Son, K. A.; Kwon, H.; Koesukwiwat, U.; Fu, W.; Mastovska, K. et al. (2010): Comparison of QuEChERS sample preparation methods for the analysis of pesticide residues in fruits and vegetables. In: *Journal of Chromatography A* 1217 (16), S. 2548–2560. DOI: 10.1016/j.chroma.2010.01.044.

Liang, C.-P.; Sack, C.; McGrath, S.; Cao, Y.; Thompson, C. J.; Robin, L. P. (2021): US Food and Drug Administration regulatory pesticide residue monitoring of human foods: 2009-2017. In: *Food Additives & Contaminants: Part A*, S. 1–19. DOI: 10.1080/19440049.2021.1934574.

Lindauer, M. (1952): A contribution to the question of the division of labor in the bee state. (Ein Beitrag zur Frage der Arbeitsteilung im Bienenstaat). In: *Z. Vergl. Physiol.* 34 (4), S. 299–345. DOI: 10.1007/BF00298048.

LTZ (2021a): Integrierter Pflanzenschutz 2021 - Ackerbau und Grünland. Online verfügbar unter https://ltz.landwirtschaft-bw.de/pb/site/pbs-bw-mlr/get/documents_E-1929596580/MLR.LEL/PB5Documents/ltz_ka/Arbeitsfelder/Pflanzenbau/Sorten/Allgemein_DL/Pflanzenschutz_DL/Integrierter%20Pflanzenschutz_Ackerbau%20und%20Gr%C3%BCnland.pdf, zuletzt geprüft am 25.03.2021.

LTZ (2021b): Integrierter Pflanzenschutz 2021_Erwerbsobstbau. Online verfügbar unter https://ltz.landwirtschaft-bw.de/pb/site/pbs-bw-mlr/get/documents_E1073610299/MLR.LEL/PB5Documents/ltz_ka/Kulturpflanzen/Obstbau/Pflanzenschutz/Integierter%20Pflanzenschutz_DL/Integrierter%20Pflanzenschutz_Erwerbsobstbau.pdf, zuletzt geprüft am 25.03.2021.

Martorell, P.; Stratford, M.; Steels, H.; Fernández-Espinar, M. T.; Querol, A. (2007): Physiological characterization of spoilage strains of Zygosaccharomyces bailii and Zygosaccharomyces rouxii isolated from high sugar environments. In: *International journal of food microbiology* 114 (2), S. 234–242. DOI: 10.1016/j.ijfoodmicro.2006.09.014.

Mauriello, G.; Prisco, A. de; Di Prisco, G.; La Storia, A.; Caprio, E. (2017): Microbial characterization of bee pollen from the Vesuvius area collected by using three different traps. In: *PloS one* 12 (9), e0183208. DOI: 10.1371/journal.pone.0183208.

McGregor, S. E. (1976): Insect Pollination Of Cultivated Crop Plants. 1. Aufl. Washington D.C.: United Department of Agriculture, zuletzt geprüft am 03.04.2020.

Meira, G. J.; Gasques, A. P.; da Silva, M. V. (2017): Food and nutrition security: pesticide residues in food. In: *Nutrire* 42 (1). DOI: 10.1186/s41110-016-0028-4.

Mekonnen, B.; Siraj, J.; Negash, S. (2021): Determination of Pesticide Residues in Food Premises Using QuECHERS Method in Bench-Sheko Zone, Southwest Ethiopia. In: *BioMed research international* 2021, S. 6612096. DOI: 10.1155/2021/6612096.

Morais, M.; Moreira, L.; Feás, X.; Estevinho, L. M. (2011): Honeybee-collected pollen from five Portuguese Natural Parks: palynological origin, phenolic content, antioxidant properties and antimicrobial activity. In: *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 49 (5), S. 1096–1101. DOI: 10.1016/j.fct.2011.01.020.

Moreno-González, D.; Alcántara-Durán, J.; Gilbert-López, B.; Beneito-Cambra, M.; Cutillas, V. M.; Rajski, L. et al. (2018): Sensitive Detection of Neonicotinoid Insecticides and Other Selected Pesticides in Pollen and Nectar Using Nanoflow Liquid Chromatography Orbitrap Tandem Mass Spectrometry. In: *Journal of AOAC International* 101 (2), S. 367–373. DOI: 10.5740/jaoacint.17-0412.

Müller, K.; Bach, M.; Hartmann, H.; Spiteller, M.; Frede, H.-G. (2002): Point- and nonpoint-source pesticide contamination in the Zwester Ohm catchment, Germany. In: *Journal of environmental quality* 31 (1), S. 309–318. DOI: 10.2134/jeq2002.3090.

Mullin, C. A.; Frazier, M.; Frazier, J. L.; Ashcraft, S.; Simonds, R.; Vanengelsdorp, D; Pettis, J. S. (2010): High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. In: *PloS one* 5 (3), e9754. DOI: 10.1371/journal.pone.0009754.

Nagai, T.; Nagashima, T.; Suzuki, N.; Inoue, R. (2005): Antioxidant Activity and Angiotensin I-Coverting Enzyme Inhibition by Enzymatic Hydrolysates from Bee Bread (60c), S. 133–138. DOI: 10.1515/znc-2005-1-224.

NCI (1978): Bioassay of lasiocarpine for possible carcinogenicity. In: *National Cancer Institute carcinogenesis technical report series* 39, S. 1–66.

Nogueira, C.; Iglesias, A.; Feás, X.; Estevinho, L. M. (2012): Commercial bee pollen with different geographical origins: a comprehensive approach. In: *International journal of molecular sciences* 13 (9), S. 11173–11187. DOI: 10.3390/ijms130911173.

Ohe, K. von der; Ohe, W. von der (2007): Celler Melissopalynologische Sammlung CMS. 3. Aufl.

Ohe, W. von der (2004): Pollen - ein wichtiger Bestandteil der Bienenernährung. Online verfügbar unter https://www.laves.niedersachsen.de/startseite/tiere/bienenkunde/informationsmaterial/bienenbiologie-bienenweide-und-bienenernaehrung-166495.html.

Ohe, W. von der (2006): Bienenbiologie - eine Einführung. Das Bieneninstitut Celle informiert. In: *LAVES - Institut für Bienenkunde Celle*.

Ohe, W. von der (2018): Pyrrolizidin-Alkaloide: Honig, Pollen, Bienen. In: *LAVES - Institut für Bienenkunde Celle*, S. 1–4, zuletzt geprüft am 08.03.2021.

Pascoal, A.; Rodrigues, S.; Teixeira, A.; Feás, X.; Estevinho, L. M. (2014): Biological activities of commercial bee pollens: antimicrobial, antimutagenic, antioxidant and anti-inflammatory. In: *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 63, S. 233–239. DOI: 10.1016/j.fct.2013.11.010.

Peris, J. (1984): Producción y comércio de los produtos apícolas en España. Apicultura 93. Bilbao.

Pesticide Properties DataBase - PPDB (2010): A to Z List of Pesticide Active Ingredients. Hg. v. Univertity of Hertfordshire. Univertity of Hertfordshire. Online verfügbar unter https://sitem.herts.ac.uk/aeru/ppdb/en/atoz.htm, zuletzt aktualisiert am April 2020.

Piffanelli, P.; Ross, J. H. E.; Murphy, D. J. (1998): Biogenessis and function of the lipidic structures of pollen grains. Review. In: *Sex Plant Reproduction* (11), S. 65–80.

Pilling, E. D.; Bromleychallenor, K.A.C.; Walker, C. H.; Jepson, P. C. (1995): Mechanism of Synergism between the Pyrethroid Insecticide λ-Cyhalothrin and the Imidazole Fungicide Prochloraz, in the Honeybee (Apis mellifera L.). In: *Pesticide Biochemistry and Physiology* 51 (1), S. 1–11. DOI: 10.1006/pest.1995.1001.

Pimentel, D. (1995): Amounts of pesticides reaching target pests: Environmental impacts and ethics. In: *Journal of Agricultural and Environmental Ethics* (8), S. 17–29, zuletzt geprüft am 22.06.2020.

Poinar, G. O.; Danforth, B. N. (2006): A fossil bee from Early Cretaceous Burmese amber. In: *Science (New York, N.Y.)* 314 (5799), S. 614. DOI: 10.1126/science.1134103.

Porter, J. H.; Parry, M. L.; Carter, T. R. (1991): The potential effects of climatic change on agricultural insect pests. In: *Agricultural and Forest Meteorology* 57 (1-3), S. 221–240. DOI: 10.1016/0168-1923(91)90088-8.

Reddy, P. V. R.; Verghese, A.; Rajan, V. (2012): Potential impact of climate change on honey bees (Apis ssp.) and their pollination services. In: *Pest Management in Horticultural Ecosystems* (18), S. 121–127, zuletzt geprüft am 22.03.2021.

Rehm, H.-J.(1980): Milchprodukte, soweit sie mit Hilfe von Mikroorganismen Hergestellt werden. In: Hans-Jürgen Rehm (Hg.): Industrielle Mikrobiologie. Zweite, völlig neubearbeitete Auflage. Berlin, Heidelberg: Springer Berlin Heidelberg, S. 578–585.

Roeder, E. (1995): Medicinal plants in Europe containing pyrrolizidine alkaloids. In: *Pharmazie* (50 (2)), S. 83–98. Online verfügbar unter https://www.researchgate.net/publication/15498141_Medicinal_Plans_in_Europe_Containing_Pyrrolizidine_Alkaloids, zuletzt geprüft am 17.02.2021.

Rortais, A.; Arnold, G.; Halm, M.-P.; Touffet-Briens, F. (2005): Modes of honeybees exposure to systemic insecticides: estimated amounts of contaminated pollen and nectar consumed by different categories of bees. In: *Apidologie* 36 (1), S. 71–83. DOI: 10.1051/apido:2004071.

Roßberg, D.; Harzer, U. (2015): Surveys on the use of pesticides in apple growing areas. Erhebungen zur Anwendung von Pflanzenschutzmitteln im Apfelanbau). In: *Journal of Crops* (67 (3)). DOI: 10.5073/JFK.2015.03.01.

Sánchez-Pérez, R.; Del, Cueto J.; Dicenta, F.; Martínez-Gómez, P. (2014): Recent advancements to study flowering time in almond and other Prunus species. In: *Frontiers in plant science* 5. DOI: 10.3389/fpls.2014.00334.

Seeburger, V. C.; D'Alvise, P.; Shaaban, B.; Schweikert, K.; Lohaus, G.; Schroeder, A.; Hasselmann, M. (2020): The trisaccharide melezitose impacts honey bees and their intestinal microbiota. In: *PloS one* 15 (4), e0230871. DOI: 10.1371/journal.pone.0230871.

Seeley, T. D. (1997): Regulation des Pollensammelns. In: Thomas D. Seeley (Hg.): Honigbienen. Im Mikrokosmos des Bienenstocks. Basel, s.l.: Birkhäuser Basel, S. 251–274.

Smith, L. W.; Culvenor, C. C. J. (1981): Plant Sources of Hepatotoxic Pyrrolizidine Alkaloids. In: *J. Nat. Prod.* (44 (2)), S. 129–152, zuletzt geprüft am 22.01.2021.

Sperber, W. H.; Doyle, M. P. (2009): Compendium of the Microbiological Spoilage of Foods and Beverages. New York, NY: Springer New York, zuletzt geprüft am 04.02.2021.

Statistisches Bundesamt (Destatis) (2019): Statistisches Jahrbuch 2019. - letztmalige Ausgabe - Titel wird eingestellt. 1., Auflage. Wiesbaden: Statistisches Bundesamt. Online verfügbar unter https://www.destatis.de/DE/Themen/Querschnitt/Jahrbuch/statistisches-jahrbuch-2019-dl.pdf;jsessionid=5D41783AC2BDD2AAFD0583366528A5A1.internet741?__blob=publicationFile, zuletzt geprüft am 10.03.2021.

Steffan-Dewenter, I.; Kuhn, A. (2003): Honeybee foraging in differentially structured landscapes. In: *Proceedings. Biological sciences* 270 (1515), S. 569–575. DOI: 10.1098/rspb.2002.2292.

Steffan-Dewenter, I.; Potts, S. G.; Packer, L. (2005): Pollinator diversity and crop pollination services are at risk. In: *Trends in ecology & evolution* 20 (12), 651-2; author reply 652-3. DOI: 10.1016/j.tree.2005.09.004.

Stoner, K. A.; Eitzer, B. D. (2013): Using a hazard quotient to evaluate pesticide residues detected in pollen trapped from honey bees (Apis mellifera) in Connecticut. In: *PloS one* 8 (10), e77550. DOI: 10.1371/journal.pone.0077550.

Stotzky, G. (1972): Activity, ecology, and population dynamics of microorganisms in soil. In: *CRC critical reviews in microbiology* 2 (1), S. 59–137. DOI: 10.3109/10408417209108383.

Tadeo, J. L. (Hg.) (2019): Analysis of pesticides in food and environmental samples. Second Edition. Second edition. Boca Raton: CRC Press, Taylor and Francis Group.

Tapparo, A.; Marton, D.; Giorio, C.; Zanella, A.; Soldà, L.; Marzaro, M. et al. (2012): Assessment of the environmental exposure of honeybees to particulate matter containing neonicotinoid insecticides coming from corn coated seeds. In: *Environmental science & technology* 46 (5), S. 2592–2599. DOI: 10.1021/es2035152.

Thakur, M.; Nanda, V. (2020): Composition and functionality of bee pollen: A review. In: *Trends in Food Science & Technology* 98, S. 82–106. DOI: 10.1016/j.tifs.2020.02.001.

Thompson, H. M.; Fryday, S. L.; Harkin, S.; Milner, S. (2014): Potential impacts of synergism in honeybees (Apis mellifera) of exposure to neonicotinoids and sprayed fungicides in crops. In: *Apidologie* 45 (5), S. 545–553. DOI: 10.1007/s13592-014-0273-6.

Throp, R. W. (2000): The collection of pollen by bees. Online verfügbar unter https://link.springer.com/chapter/10.1007/978-3-7091-6306-1_11.

Traynor, K. S.; Pettis, J. S.; Tarpy, D. R.; Mullin, C. A.; Frazier, J. L.; Frazier, M.; Vanengelsdorp, D. (2016): In-hive Pesticide Exposome: Assessing risks to migratory honey bees from in-hive pesticide contamination in the Eastern United States. In: *Scientific reports* 6, S. 33207. DOI: 10.1038/srep33207.

Umweltbundesamt (2020): Pflanzenschutzmittelverwendung in der Landwirtschaft. Hg. v. Umweltbundesamt. Online verfügbar unter https://www.umweltbundesamt.de/daten/land-forstwirtschaft/pflanzenschutzmittelverwendung-in-der#zulassung-von-pflanzenschutzmitteln.

van der Steen, J.J.M. (2015): The Foraging Honey Bee. In: *The British Bee Journal*, S. 43–46. Online verfügbar unter https://library.wur.nl/WebQuery/wurpubs/fulltext/335934.

van Engelsdorp, D.; Caron, D.; Hayes, J.; Underwood, R.; Henson, M.; Rennich, K.et al. (2012): A national survey of managed honey bee 2010–11 winter colony losses in the USA: results from the Bee Informed Partnership. In: *Journal of Apicultural Research* 51 (1), S. 115–124. DOI: 10.3896/IBRA.1.51.1.14.

VO (EU) 2018/ 62: VERORDNUNG (EU) 2018/ 62 DER KOMMISSION - vom 17. Januar 2018 - zur Ersetzung des Anhangs I der Verordnung (EG) Nr. 396/ 2005 des Europäischen Parlaments und des Rates, zuletzt geprüft am 10.03.2021.

Wallner, K. (2012): Not in the bloom. (Nicht in die Blüte). In: *German bee journal* (9/2012), S. 18–19. Online verfügbar unter https://link.springer.com/content/pdf/10.1007/s13592-017-0551-1.pdf, zuletzt geprüft am 16.04.2020.

Wernecke, A.; Frommberger, M.; Forster, R.; Pistorius, J. (2019): Lethal effects of various tank mixtures including insecticides, fungicides and fertilizers on honey bees under laboratory, semi-field and field conditions. In: *J Consum Prot Food Saf* 14 (3), S. 239–249. DOI: 10.1007/s00003-019-01233-5.

Wilde, J. (1991): Einfluß der Einsatzdauer einer Pollenfalle auf die Pollenernte und Entwicklung von Bienenvölkern. In: *Apidologie* (22). Online verfügbar unter https://ibra.org.uk/wp-content/JAAS/VOL2/2-4/JAAS%202%204%2001L%20%25281%2529.pdf.

Wilkowska, A.; Biziuk, M. (2011): Determination of pesticide residues in food matrices using the QuEChERS methodology. In: *Food chemistry* 125 (3), S. 803–812. DOI: 10.1016/j.foodchem.2010.09.094.

Willmer, P. (2011): Pollination and floral ecology. New Jersey: Princeton University Press.

Winston, M. L. (1987): The Biology of the Honey Bee.

Yun, J.-H.; Jung, M.-J; Kim, P. S.; Bae, J.-W. (2018): Social status shapes the bacterial and fungal gut communities of the honey bee. In: *Scientific reports* 8 (1), S. 2019. DOI: 10.1038/s41598-018-19860-7.

Zalar, P.; Hoog, G. S. de; Schroers, H-J; Crous, P. W.; Groenewald, J. Z.; Gunde-Cimerman, N. (2007): Phylogeny and ecology of the ubiquitous saprobe Cladosporium sphaerospermum, with descriptions of seven new species from hypersaline environments. In: *Studies in mycology* 58, S. 157–183. DOI: 10.3114/sim.2007.58.06.

Danksagungen

Die Arbeit wurde durch das Ministerium für Ländlichen Raum und Verbraucherschutz Baden-Württemberg finanziell unterstützt.

Einen großen Dank möchte ich an Prof. Dr. Martin Hasselmann richten, für die engagierte Betreuung und die Unterstützung bei der Datenauswertung meiner Promotionsarbeit. Seine positive Einstellung und guten Zusprüche während der Promotion, haben mich nie aufgeben und schlussendlich meine Arbeit erfolgreich abschließen lassen.

Prof. Dr. Walter Vetter möchte ich für seine Betreuung und Unterstützung während meiner Promotion danken und seine forcierte Herangehensweise die Ergebnisse meiner Arbeit zu publizieren.

Prof. Dr. Jan Frank danke ich recht herzlich für sein spontanes Einverständnis zur Übernahme der Zweitbegutachtung und sein Interesse an der vorliegenden Arbeit.

Vielen Dank an PD Dr. Peter Rosenkranz für die Bereitstellung der Laboratorien und seine wertvollen Hinweise bei der Durchführung der Versuche und Verfassung der Manuskripte.

Mein besonderer Dank gilt Dr. Klaus Wallner, ohne den diese Arbeit nicht möglich gewesen wäre. Ich danke ihm für seine positive Einstellung während der Betreuung, seine wertvollen Ratschläge und besonders, dass er immer ein offenes Ohr für mich hatte.

Darüber hinaus möchte ich mich bei Dr. Paul D'Alvise und Dr. Karsten Schweickert für ihre hilfreichen Ratschläge bei der Auswertung von Ergebnissen bedanken.

Dr. Dieter Martens und Thomas Kapp danke ich für die Analysen der zahlreichen Pollenproben.

Danke an Dr. Raghdan Alkattea für die Unterstützung bei der palynologischen Analyse und die unzähligen gemeinsamen Stunden hinter dem Mikroskop.

Ich danke allen Imkerinnen und Imkern, die sich an den Sammelversuchen der Arbeit beteiligt und mit großer Begeisterung mitgewirkt haben.

Bedanken möchte ich mich außerdem bei meinen lieben Kolleginnen und Kollegen der Landesanstalt für Bienenkunde sowie der Populationsgenomik, durch die ich immer in einem sehr angenehmen und positiven Arbeitsklima arbeiten durfte.

Einen lieben Dank gilt besonders Birgit Fritz, die mir jede noch so schwere Rechnung in einem Dreisatz erklären konnte und mir in den letzten Jahren mit ihrer warmherzigen und hilfsbereiten Art immer den Rücken gestärkt hat und zu jeder Zeit eine verlässliche Ansprechpartnerin war.

Vielen Dank an meine Kolleginnen Dr. Bettina Ziegelmann, Melanie Marquardt und Carolin Rein für die großartige Arbeitsatmosphäre und die netten Gespräche.

Besonders möchte ich meiner lieben Kollegin und Zimmernachbarin Dr. Victoria Seeburger danken, die mich durch ihre wundervolle Persönlichkeit immer zum Lachen gebracht und sich dadurch zu einer unersetzbaren Freundin für mich entwickelt hat.

Unendlich dankbar bin ich meinem Partner Maximilian, für seine bedingungslose Unterstützung und Geduld, sowie seinen unbeirrten Glauben an mich.

Nicht zuletzt möchte ich meiner Familie danken, die mich in all meinen Vorhaben unterstützt und durch ihren Stolz motiviert hat an meinen Träumen festzuhalten.

Lebenslauf

Persönliche Daten

Name	Carolin Gertrud Maria Friedle
Geburtsdaten	29.07.1993 in Stuttgart

Berufserfahrung

seit 05/2021	**Manager Regulatory Affairs**
	Weckerle Cosmetics GmbH, Schillerstraße 23-25, 73054 Eislingen/Filz
	Fachliche Leitung des Bereichs Regulatory Affairs
04/2018 – 04/2021	**Wissenschaftliche Mitarbeiterin**
	Landesanstalt für Bienenkunde, Universität Hohenheim
	Projektleitung und -Verwaltung; Akademische Lehre; Bildungsdidaktik
07/2017 – 03/2018	**Wissenschaftliche Hilfskraft**
	Landesanstalt für Bienenkunde, Universität Hohenheim
	Rückstandsanalytik von Bienenprodukten
02/2017 – 03/2017	**Forschungspraktikum**
	Massey Universität Auckland, Neuseeland
	Mitarbeit bei einer klinischen Studie, Degustation, Datenauswertung

Ausbildung

seit 04/2018	**Promotion an der Fakultät für Naturwissenschaften**
	Landesanstalt für Bienenkunde, Universität Hohenheim
	Bereich für Lebensmittelchemie und Analytik
10/2015 – 03/2018	**Master of Science; Lebensmittelchemie**
	Universität Hohenheim; Stuttgart-Hohenheim
08/2016	**Ausbildung zur Qualitätsmanagement Fachkraft**
	TÜV – SÜD Akademie
10/2012 – 09/2015	**Bachelor of Science; Lebensmittelchemie**
	Universität Stuttgart, Stuttgart-Vaihingen

07/2012 **Abitur**

Wilhelms-Gymnasium Degerloch, Stuttgart

Liste der Veröffentlichungen

Friedle, C., Wallner, K., Rosenkranz, P., Martens, D., Vetter, W. (2021): Pesticide residues in daily bee pollen samples (April-July) from an intensive agricultural region in Southern Germany. In: *Environ Sci Pollut Res,* S. 1-15. DOI: 10.1007/s11356-020-12318-2.

Friedle, C.; D'Alvise, P.; Schweikert, K.; Wallner, K.; Hasselmann, M. (2021): Changes of microorganism composition in fresh and stored bee pollen from Southern Germany. In: *Environ Sci Pollut Res*. DOI: 10.1007/s11356-021-13932-4.

Friedle, C., Kapp, T., Wallner, K., Alkattea, R., Vetter, W. (2022): High abundance of pyrrolizidine alkaloids in bee pollen collected in July 2019 from Southern Germany. In: *Environ Moni and Ass.* DOI: 10.1007/s10661-022-09907-8.

Liste der Tagungsbeiträge

03/2018 65. Tagung der AG der Institute für Bienenforschung e.V. in Koblenz

Posterbeitrag mit dem Thema: „Entwicklung und Validierung eines schnellen Analyseverfahrens zur Messung von Pflanzenschutzmittelrückständen im Nektar heimkehrender Bienen mittels GC-ECD und LC-MS/MS“

03/2018 47. Deutsche Lebensmittelchemiker Regionaltagung (GDCh) in Gießen

Posterbeitrag mit dem Thema: „Entwicklung und Validierung eines schnellen Analyseverfahrens zur Messung von Pflanzenschutzmittelrückständen im Nektar heimkehrender Bienen mittels GC-ECD und LC-MS/MS“

03/2019 66. Tagung der AG der Institute für Bienenforschung e.V. in Frankfurt

Posterbeitrag mit dem Thema: „Charakterisierung der Qualität von Blütenpollen“

09/2019 48. Deutsche Lebensmittelchemiker Regionaltagung (GDCh) in Dresden

Posterbeitrag mit dem Thema: „Untersuchung der mikrobiellen Zusammensetzung von Blütenpollen“

Fellbach, 06.03.2022 C. Friedle

Ort und Datum Unterschrift

Eidesstattliche Versicherung

gemäß §7 Absatz 7 der Promotionsordnung der Universität Hohenheim zum Dr. rer. nat.

1. Bei der eingereichten Dissertation zum Thema
   „Charakterisierung der Qualität von Blütenpollen in unterschiedlichen Regionen Baden-Württembergs“
   Handelt es sich um meine eigenständig erbrachte Leistung.

2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.

3. Ich habe nicht die Hilfe einer kommerziellen Promotionsvermittlung oder -beratung in Anspruch genommen.

4. Die Bedeutung der eidesstattlichen Versicherung und der strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung sind mir bekannt.

Die Richtigkeit der vorstehenden Erklärung bestätige ich: Ich versichere an Eides statt, dass ich nach bestem Wissen die reine Wahrheit erklärt und nichts verschwiegen habe.

Fellbach, 06.03.2022 — C. Friedle

Ort und Datum — Unterschrift