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CORN SEED COATED WITH NEONICOTINOIDS: ENVIRONMENTAL CONTAMINATION AND BEE LOSSES IN SPRING

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“La polemica è
sempre nociva al
polemista, ma spesso è utile
alla comunità”

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31 January, 2013 Matteo Marzaro

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Table of content

Riassunto

Negli ultimi dieci anni si è assistito, a livello nazionale ed internazionale, ad una crisi nel settore dell'apicoltura dovuto a morie di api che portano spesso ad un completo spopolamento degli alveari. Questo fenomeno ha assunto intensità particolarmente gravi nel triennio 2005-2008, con perdite fino al 40% del totale degli alveari produttivi in alcuni stati europei, tra cui l'Italia e sino al 40-60% negli Stati Uniti. Questa generalizzata moria delle api, denominata "Colony Collapse Disorder" (CCD) ha comportato una serie di interrogativi sulle cause, tra le quali la diffusione di parassiti come la *Varroa destructor* oppure il *Nosema spp.*, le tecniche apistiche sempre più spinte e il diffuso inquinamento ambientale dovuto in gran parte all'uso di prodotti fitosanitari. In particolare, la perdita di colonie che si osserva in autunno è dovuta nella generalità dei casi alla varroa, mentre le perdite primaverili, osservate prima del bando dei neonicotinoidi nel 2008, avvengono in corrispondenza delle semine primaverili del mais, dove si impiegano sementi conciate con insetticidi appartenenti alla famiglia dei neonicotinoidi e seminatrici pneumatiche.

In questa tesi si sono approfonditi gli aspetti che riguardano la moria delle api, soprattutto in riferimento al periodo primaverile. Si possono infatti distinguere le cause che differenziano le perdite delle colonie nel periodo autunnale provocate essenzialmente dall'acaro *Varroa destructor*, dalle perdite primaverili, le cui cause, pur essendo associate alle semine del mais con neonicotinoidi, erano praticamente sconosciute nel modalità di intossicazione sino all'inizio della presente tesi; si attribuiva infatti la morte delle api a dosi sub letali acquisite dalla vegetazione spontanea circostante i seminativi. Si è considerata invece l'ipotesi che esistessero fonti di intossicazione acuta legate alla semina del mais. Si sono ipotizzati due diversi meccanismi d'azione nell'avvelenamento delle api. Il primo consiste nel rilascio, sottoforma di gocce, attraverso un fenomeno fisiologico chiamato guttazione, di ingenti quantità di principio attivo con le quali le api potrebbero venire a contatto; l'altro meccanismo considera l'intossicazione da insetticidi attraverso la semina di mais conciato con la liberazione nell'aria di particolato contenente concentrazioni altissime di insetticida con il quale le api si intossicano in volo.

Le giovani piante che sviluppano una pressione radicale elevata, mostrano frequentemente la fuoriuscita di liquido dai margini delle foglie, un fenomeno detto guttazione. Le "gocce di rugiada" osservate sulle foglie delle graminacee al mattino sono

per lo più gocce di guttazione, in particolare se presenti sulla punta delle foglie. Le gocce sono prodotte e permangono sulla lamina delle foglie delle piantine di mais anche per parecchie ore, ma possono cadere o asciugarsi rapidamente in presenza di vento. Nel calice, la guttazione permane anche per tutto il giorno. Tali gocce durante il periodo primaverile possono venir utilizzate dalle api come fonte idrica anche per umettare l'alveare.

Nei primi due lavori presenti in questa tesi sono illustrati i risultati del primo anno dell'attività di dottorato, cioè la scoperta che le gocce di guttazione contengono concentrazioni elevate, anche centinaia di ppm, di insetticidi (neonicotinoidi) impiegati nella concia dei semi: queste molecole essendo idrosolubili entrano in circolo nella pianta e sono quindi parzialmente rilasciati attraverso le guttazioni. Tali concentrazioni, se si considera la capacità di ingestione di un'ape (stimata attorno a 20-30 µl), risultano ben al di sopra della dose letale sia per ingestione ed anche per contatto (circa 20-40 ng di p.a./ape). Si è tuttavia esclusa l'implicazione delle guttazioni nelle catastrofiche morie primaverili attraverso osservazioni di campo che hanno messo in luce come tali gocce non costituiscano di norma una fonte idrica per le api a meno che, si suppone, non perduri un lungo periodo di siccità.

Nei lavori successivi si è quindi considerata l'ipotesi che l'effetto tossico dei neonicotinoidi usati per la concia del seme di mais, fosse direttamente connesso all'esposizione delle api alle polveri emesse durante la semina. Si sono svolte prove in campo, sia per quanto riguarda l'emissione del particolato, sia sul meccanismo e i fattori che provocavano un intossicazione letale alle api.

Dalle analisi chimiche (eseguite dal Dipartimento di Scienze Chimiche dell'Università di Padova), le quantità di insetticidi rinvenuti su api morte, anche dopo un solo volo vicino alla seminatrice, sono comprese tra circa 50 e 1000 ng per ape, con una punta massima riscontrata pari a 11 µg/ape.

Parallelamente alle prove in campo, si è provveduto a quantificare con maggior precisione la quantità di polvere che viene emessa dalla seminatrice (sesto lavoro) e contemporaneamente a determinare la dimensione della "nube tossica" formata dal particolato proiettato in aria durante la semina. Nel quinto lavoro presente nella tesi, è stata dimostrata così la presenza di una nube tossica attorno alla macchina, che in assenza di vento sostenuto, ha la forma di un elissoide di circa 20 metri di diametro e di almeno 3 metri d'altezza. Tra i vari risultati ottenuti è rilevante sottolineare anche la messa a punto un semplice test biologico per saggiare l'effettiva intossicazione delle api in vicinanza

delle seminatrici i cui risultati sono in ottimo accordo con i dati analitici di emissione e/o di ricaduta delle particelle originate con la semina.

Le analisi chimiche, in particolare, hanno consentito di mettere a punto un protocollo innovativo per la determinazione di insetticidi neonicotinoidi in singole api (settimo lavoro); sino ad ora tutte le analisi riportate in letteratura riguardano l'esame di campioni di almeno alcune decine di api (es. kg di insetti morti). Ne consegue che l'analisi chimica più raffinata permette di quantificare la contaminazione del singolo insetto con un evidente vantaggio per la comprensione e valutazione delle cause di intossicazione.

Summary

The last ten years have witnessed, both at a national and an international level, a crisis in the beekeeping sector due to the death of bees often causing a complete depopulation of hives. This phenomenon has assumed a particularly serious intensity in the three years 2005-2008 with a loss of up to 40% of hives in some states in Europe, amongst these Italy, and losses up to 40-60% in the United States. The name "Colony Collapse Disorder has been coined to describe these generalised bee deaths and a number of questions have been raised as to the causes, amongst these are the spread of parasites such as *Varroa destructor* or *Nosema spp.*, ever more extreme beekeeping techniques and the widespread environmental pollution due in great measure the use of phyto-sanitary products. The colony losses that have been observed in the Autumn in particular, are generally due to the instance of varroa, while the Spring losses, before the banning of neonicotinoids in 2008, appeared at the same time as the Spring sowing of maize seed where seed coated with insecticide of the neonicotinoid family were sown using pneumatic seed drills.

This text studies in depth aspects which are concerned with the death of bees, particularly in the Spring period. It is possible, in fact, to distinguish the causes which differentiate the losses in the Autumn, caused essentially by the bee mite *Varroa destructor* from the Spring losses the cause of which is associated with the sowing of maize with neonicotinoids. The means by which poisoning occurred were virtually unknown until the start of the present thesis. Up to this point the deaths had in fact been attributed to poisoning by sub-lethal doses which bees had picked up from the self-sown vegetation surrounding the sowing areas. Instead, the hypothesis has been considered that there exist sources of acute poisoning connected to the sowing of maize. Two different poisoning mechanisms were hypothesised. The first consisted of the release of droplets containing substantial quantities of active ingredient with which the bees could come into contact through a physiological phenomenon called guttation; the second mechanism considered was poisoning with insecticide through the sowing of coated maize seed, whereby large quantities of dust, containing high concentrations of insecticide, poisoned the bees in flight.

Young plants which develop raised root pressure frequently show an emission of liquid around the edge of the leaves, a phenomenon called guttation. The "drops of dew" seen on the leaves of graminaceous plants in the morning are, for most part, guttation drops, in

particular on the points of the leaves. The drops are produced and remain on the lamina of the leaves of maize seedlings, often for some hours, but they can drop off or dry out rapidly if wind is present. Guttation can even remain in the calyx of the plant for the whole day. During the Spring period such drops can be used by bees as a water source with which to moisten the hive

The first two studies in this thesis illustrate and show the results of the first year of doctoral activity, that is the discovery that the droplets of guttation contain elevated concentrations, some hundreds of ppm, of inscticides (neonicotinoids) employed in the seed coating. This insecticide, being systemic (water soluble), enters into the circulation of the plant, and is thus released through guttation. Such concentrations, if we consider a bees capacity to ingest (estimated around 20-30µl), proves to be well above the lethal dose for ingestion or, even for contact (ca. 20-40µl of a.i. per bee). However, the implication of guttation in the catastrophic Spring deaths has been excluded through the observations in the field, in which it came to light that such droplets do not constitute a normal water source for bees, unless we assume a long period of drought.

Therefore, in subsequent studies the hypothesis was considered that the toxic effects of neonicotinoids used for the coating of maize seed could be directly related the exposure of bees to the dust emitted during the maize sowing. Field trials were undertaken, both with regard to the emission of particulates, and to determine the mechanisms and the factors that caused the lethal poisoning of bees.

From chemical analysis (conducted by the Department of Chemistry of the University of Padua) it was shown that, even after a single flight in the vicinity of the seed drill, that quantities from between about 50 and 1000 ng per bee were present with a maximum encountered equal to 11µg/bee. In parallel with the field trials steps were taken to quantify the amount of dust emitted by the seed drill with great precision (six trials), and at the same time to determine the dimensions of the "toxic cloud" made up of particulates projected into the air during the sowing. In the fifth study in this thesis, the presence of a toxic cloud around the machine was demonstrated which, in the absence of a sustained wind, had an ellipsoidal form of approximately 20 metres in diameter and at least 3 metres in height. Among the various results obtained, it is also important to underline the setting up of a simple biological test to establish the toxic effect to bees flying near the seed drill. These results are in precise agreement with the analytical data of emissions and/or the fall of particulates emanating from the seed drill.

The chemical analysis, in particular, allowed the putting on place of an innovative protocol to determine the amount of neonicotinoid insecticide in a single bee (seventh study); up to that point all the analysis reported in the literature concerned the examination of at least several hundreds of bees (for example kg of dead insects). It follows that the more refined chemical analysis allows the quantification of contamination in a single insect with an evident advantage in the understanding and evaluation of the cause of poisoning.

Introduction.

Beekeeping in Europe is an ancient tradition, as it is throughout the world and bees have been reared through the millennia (Moritz *et al.*, 2010; Ransome, 1937). Bees are a fundamental necessity for the environment in that they favour biodiversity and have an essential role in pollination (Steffan-Deweter *et al.*, 2005; Steffan-Deweter *et al.*, 2006; Klein *et al.*, 2007) of many plants, both cultivated and wild (Costanza *et al.*, 1997). They contribute directly to the wealth and the well-being of man in the production of honey and other products, for example pollen, beeswax in the manufacture of foodstuffs, propolis for food technology and royal jelly as food ingredient and diet adjunct. The greater part of agriculture in the European Union depends upon pollination by insects (Gallai *et al.*, 2009). In fact, according to estimates from the United Nations Food and Agriculture Organisation (FAO), of the 100 types of food culture that furnish 90% of the world's food, 71 are pollinated by bees (Delaplane and Mayer, 2000; Cane and Schiffhauer, 2003; Aizen *et al.*, 2008; Aizen *et al.*, 2009; Winfree *et al.*, 2011; Calderone, 2012). Moreover, beyond bees' fundamental value in pollination and maintenance of biodiversity (Butchart *et al.*, 2010), they contribute an estimated global monetary value of hundreds of millions of euro (€14.4 billion) (Gallai *et al.*, 2009). Therefore, in the light of the scale of the ecological and economic value of bees, it is essential to monitor and maintain reserves of healthy bees, not only on a local or national level, but on a global level (Huang, 2012).

In the last few years, pollinators, but in particular honeybee (colonies), throughout the world have been subject to rapid losses (Stokstad, 2007; Biesmeijer *et al.*, 2006; Ellis *et al.*, 2010; Potts *et al.* 2010). The beehive heritage in Europe decreased from over 22.5 million in 1990 to about 15.5 million in 2009 (FAO 2011). From a European perspective, the countries particularly concerned over the losses of bees, are France (Chauzat *et al.*, 2010), Belgium and Switzerland (Charrière and Neumann, 2010), Germany (Genersch, 2010/a), the United Kingdom (Gray *et al.*, 2010), the Netherlands (van der Zee, 2012), Italy (Mutinelli *et al.*, 2010) and Spain (Bernal *et al.*, 2010). In North America, the observed loss of colonies since 2005 has left some states, struck by the unusual death rate, with the lowest number of bee colonies ever registered in the last 50 years (vanEngelsdorp *et al.* 2007;vanEngelsdorp *et al.*, 2010). American studies have coined the expression *Colony Collapse Disorder* or CCD to describe this phenomenon of apiary depopulation. CCD is characterized by 1) the sudden reduction of adult bees in the colony with only a few remaining; 2) the presence of many unaltered opercula brood cells and a

low level of varroa infestation, which indicates that the colony had been relatively strong before the loss of adult bees, and that the collapse of the colony cannot be attributed to infestation of varroa mite; 3) the reserves of food indicate that no raiding has taken place even though there are active neighboring colonies; 4) the minimal presence of the small hive beetle *Aethina tumida* furthermore, the frequent presence of an egg laying queen bee surrounded by small groups of feeding young (vanEngelsdorp, 2009).

No single cause of the loss of numbers has been pinpointed (Genersch, 2010b; Le Conte *et al.*, 2010; vanEngelsdorp and Meixner, 2010; Neumann and Carreck, 2010). The different phenomena of death and depopulation simple show some common elements ascribable to CCD but do not permit the hypothesizing of an unequivocal explanation, be it concerning the problems of survival and development of the bees, be it with reference to the various causes of stress or mortality. The limited and fragmentary nature of our knowledge of the differing phenomena of bee mortality notwithstanding, it is possible to ascertain that in various geographical areas where deaths occurred, the phenomena showed specific characteristics; it is sufficient to note, for example, that the abandoned brood of the bee colonies attracted no predators (or pillaging activity in food stocks abandoned). The same codification CCD, as defined in American research (Underwood and vanEngelsdorp, 2007) to analyze the phenomenon of deaths in the U.S.A., while it is persuasive and plausible, cannot explain all the deaths and difficulties encountered in apiaries throughout the world. The superficiality and the tendency to arrive at easy and unjustifiable generalizations that are encountered in the substantial literature that has developed around the phenomenon of bee deaths, raises doubts that the inferences as to cause and explanation are properly identifiable. When we find ourselves confronted with signs of manifest environmental imbalance resulting from intense production or economic activity, the analyses that are often conducted, particularly those of a scientific nature, are not entirely neutral. Powerful smokescreens are established to obscure the real cause of these phenomena, and sometimes, what is worse, hypotheses are suggested, the sole character of which, is to mystify and mislead. Diverse concomitant factors are suggested, which could cause possible stress or have an immune-depressive effect on bees, which are arrived at through one, of a combination of these factors. At first, whether at a national (in Spain - Higes *et al.*, 2006; Higes *et al.*, 2009) or international level, it seemed the depopulation could be caused by fungi, for example the new Asiatic species of *Nosema,* the *Nosema ceranae* (Fries *et al.*, 1996; Paxton *et al.*, 2007; Chen *et al.*, 2008). This new specie, along with already existing *Nosema apis* (Zander, 1909) was believed to

be the most important factor in bee deaths in all other areas with CCD symptoms. On that point, Genersch (2010), cited that attacks of *Nosema* spp. *...killing of honey bee colonies might be a regional problem rather than a global phenomenon"*. Other stress factors taken into consideration were food of poor nutritional quality due to foraging among crops with low nutritional value or, again a lack of pollen and nectar due to the increasing reduction in biodiversity of cultivated areas (Naug, 2009). Drought is also taken into consideration and the unscrupulousness of beekeeping practices, in particular "nomadism" (Genersch, 2010/b), caused by the necessity to continuously relocate in search of flowers.

Numerous evaluations have also looked at viruses as a pathogenic agent capable of causing substantial damage to the bee heritage of the states concerned. Research in the United States, in particular is directed at identifying the cause of CCD by means of a genetic approach. The metagenomics allows the simultaneous study of the genomes of all the micro-organisms present in a particular environment, and enables the identification of new species present. This approach has allowed American researchers to quickly establish a census of the micro flora of colonies, both affected and unaffected by the syndrome, furnishing a basis for a valuation of the significance and the provenance of possible pathogenic agents (Cox-Foster *et al.,* 2007). The prevalence in the sequences of samples taken from affected colonies of the Israel Acute Paralysis Virus (IAPV) has been observed (Chen and Evans, 2007), as has a correlation between the presence of the virus and bee deaths. Similar considerations concerned, according to Highfield *et al.* (2009) the Deformed Wing Virus (DWV).

The same authors (Cox-Foster e vanEngelsdorp, 2009), have successively demonstrated the marginal effect of the presence of the virus alone in determining such depopulation, as that cited above.

This consideration was reinforces in a study conducted in the North of Italy by ISPRA (Istituto Superiore per la Protezione e la Ricerca Ambientale) (2010) which confirmed that *"the pathology, when considered alone (Nosema spp. and virus), although present, determined neither the phenomenon of acute bee death nor the depopulation and loss of apiaries"*.

The bee parasite most recognised by everyone throughout the world as the cause of substantial colony losses is (Thompson *et al.* 2002; Rosenkranz *et al.,* 2010) the hematophagous mite *Varroa destructor* A&T (Anderson and Trueman, 2000). During the first half of the last century, in particular from the 1970s to the 1990s, *V. destructor*

appeared in Europe and United States (Anderson, 2000), passing from the East Asiatic *Apis cerana* Fabricious to the African and European *Apis mellifera* L (Anderson and Trueman, 2000), causing a dramatic decrease in the number of apiaries and beekeepers (Neumann *et al.* 2010; Ratnieks *et al.* 2010). The individual honey bee is damaged in many ways, for example the hatching bee has an average loss of body weight, decreased flight performance in drones, transmission of viruses, moreover, worker bees parasitized (during their development) start earlier with foraging with consequent reduced life span and decreased capability of non-associated learning. At colony level, the damages, are less numbers of swarms (Fries *et al.*, 2003; Villa *et al.*, 2008), a lower chance to mate for drones and in general, the reduction of bee population (Shimanuki *et al.*, 1994). Moreover, the untreated colonies which exceed an infestation rate of about 30% in the adult bees during the summer do not have a chance to survive the following winter (Fries *et al.*, 2003; Rosenkranz *et al.* 2006). For these reasons, colonies infected by varroa die within the space of 1-3 years without chemical interventions (vanEngelsdorp *et al.* 2008; Rosenkranz *et al.* 2010; vanEngelsorp and Meixner, 2010), which are effective in reducing the losses that are observed in Autumn and the end of Winter (Kraus and Page 1995; Fries and Perez-Escala, 2001). In addition, the correlation between virus-detection, varroa infestation level and colony mortality is not as clear as expected and demonstrates the need of a standardized quantitative virus analysis under field conditions (Rosenkranz *et al.* 2010).

Analyzing the evolution of thought relative to the literature available, it is possible to see that varroa, which in the early studies on CCD, appeared to be a concomitant cause (amongst other things it was reported that the depopulated broods did not often present with infestation consistent with varroa), subsequently became the principal cause and *Nosema* spp. and environmental and feeding factors assumed a secondary importance.

Pesticides used in agriculture for insect and mite control are often involved in cases of bee mortalities, it is recognized that they can kill many beneficial insects and the residues of such active ingredients can be found both in the bodies of dead adult honey bees, and in hive products (Porrini *et al.*, 2003; Frazier *et al.*, 2008; Johnson *et al.*, 2010). In agro-ecosystems pesticides are applied in different environmental conditions, with different application technologies and concentrations. Soil insecticides were applied (during sowing) for the control of common soil insects like wireworm beetles (*Agriotes* spp.) and cutworms (*Agrotis* spp.) but also against rootworm, *Diabrotica virgifera* LeConte (Stamm *et al.*, 1985; Altmann, 2003; van Rozen and Ester, 2010).At the end of the 1990s,

soil insecticides were replaced by the coating of (maize) seeds (Taylor and Harman, 1990), of the modern active ingredients employed, the first was fipronil (Colliot *et al.*, 1992; Turnblad 1998) and more recently, neonicotinoids (Elbert *et al.*, 2008), in particular imidacloprid (Elbert et al, 1990), thiamethoxam (Maienfisch *et al.*, 2001; Robinson 2001) and clothianidin (Altmann, 2003; Andersch and Schwarz, 2003). Upon application of neonicotinoid to the seed surface, the insecticides (by virtue of the high systemic properties of their molecules) are transferred and distributed throughout the whole plant, conferring a substantial and long-lasting control of insects. The high systemic activity, as conferred to the seedling, is a protection against sucking leafhoppers and aphids (Magalhaes *et al.*, 2009). A new revolutionary application was the effective limitation of virus transmission (Elbert *et al.*, 2008;Jeschke *et al.*, 2010). In this way the sensitiveness to the virus of possible new hybrids (or varieties) has benefitted both producers and farmers.

Recently, the insecticides employed in coating seed have become the primary suspects in the lethal Spring poisoning that has caused the losses of apiaries seen to occur at the same time as the sowing of the maize (*Zea mais*L), from mid-March to May, in the corn growing regions or northern Italy and Europe, and is distinguishable from the Winter losses caused by varroa (Girolami *et al.*, 2012). On a global level the association of the death of bees with seeds coated with neonicotinoids could be considered to have been born in France with the hypothesis that the flowers of the sunflowers were thought to have remained poisoned with the insecticide rising from the seeds sown months earlier. The amount of neonicotinoid insecticides in nectar and pollen has been reported in the order of parts per billion (ppb) (Schmuck, 1999; Schmuck *et al.*, 2001; Laurent and Rathahao, 2003; Bonmatin *et al.*, 2005). The connection between the neonicotinoid shell of seed, the death of bees, the implication that abraded particles of the insecticide shell expelled from the pneumatic sowing machines consequently fell on, poisoning the surrounding vegetation causing the catastrophic death of bees (at the same time as the sowing of maize), had originated in Italy at the end of the 90s' (Greatti *et al.* 2003). In the case of maize, as in the earlier case of sunflowers in France, the implication of neonicotinoids in the poisoning of bees was immediately negated (Schnier *et al.*, 2003; Chauzat *et al.*, 2006).

The hypothesis took account of the sub-lethal effects caused by the insecticide dust emitted by the sowing drill because of the systemic properties of neonicotinoids (Greatti

et al., 2003; Greatti *et al.*, 2006, Maini *et al.*, 2010). Nevertheless, the amount of insecticides found in vegetation did not seem to justify such rapid losses during, or immediately after sowing, since the insecticide content is about 50 ppb (Greatti *et al.*, 2006). That seemed too low a dose to cause poisoning by ingestion according to Yang *et al.* (2008), even if sub-lethal effects over the long period were considered (Colin *et al.*, 2001; Suchail *et al.*, 2001; Bortolotti *et al.*, 2003; Colin *et al.*, 2004; Decourtye *et al.*, 2004; Medrzycki *et al.*, 2003; Maini *et al.*, 2010; Laurino *et al.*, 2011). Another element whereby these sub-lethal doses did not seem to justify the deaths is illustrated by the description of symptoms in the literature on the subject. Such symptoms of such doses, loss of memory and the capacity to orientate, supported the theory of the disorientation of foragers (Sgolastra *et al.*, 2012; Teeters *et al.*, 2012) which could not succeed in regaining the hive, thus depopulating the apiaries. The implications of sub-lethal doses in the sudden deaths of bees in front of the hives (during the maize sowing season) is called into question also by the chemical analyses, given that half of the samplings showed negative (below the detectable level) and others showed only traces. Few of the samples tested positive, with doses that would allow for an acute poisoning (Bortolotti *et al.*, 2008).

This contrasts with the accumulations of dead bees frequently found in the case of the springtime deaths caused by neonicotinoids, for these reasons other lethal sources in the fields were sought to justify such rapid mortality during the spring maize sowing.

Objectives and content of the thesis

The objectives of the thesis were an in-depth analysis of the knowledge of some aspects concerning the death of bees, particularly with reference to the Spring period. One can in fact identify the causes that differentiate the colony losses in the Autumn period, occasioned essentially by the bee mite *Varroa destructor* A&T, from the Spring losses whose cause is associated simply with the Spring sowing of maize seed coated with neonicotinoids (Greatti *et al.*, 2003; Greatti *et al.*, 2006). The causes, and the means by which bees were poisoned, were virtually unknown up to the commencement of this current thesis, and were in fact, attributed to bees dying from sub-lethal doses acquired from the self-sown vegetation surrounding the sown fields. The hypothesis was considered that sources of acute poisoning existed, linked to the maize sowing. Two different means of the poisoning were identified which in turn, identified the two lines of research found in this thesis.

The first consists of the release by young maize plants of tiny droplets, through a physiological phenomenon called guttation (Goatley and Lewis, 1966; Hughes and Brimblecombe, 1994), containing considerable quantities of the active ingredient with which bees could come into contact. The concentrations of neonicotinoids in the droplets were studied by chemical analysis and agronomic and environmental factors which could influence the insecticide content were taken into account. Then the toxicity of these droplets was assessed to demonstrate that the presence of such droplets on the vegetation (maize seedlings) could present a potentially lethal risk for the bees. In collaboration with a working group of Professor Tapparo of the Department of Chemistry at the University of Padua, a pre-existing method of analysis (UHPLC-DAD) (Guzsvany *et al.*, 2006; Zhou *et al.*, 2006) was optimized and perfected to enable the analysis of the insecticide in guttation rapidly, and with greater precision than methodologies previously used.

The second mechanism considered, envisaged the poisoning of bees in flight by direct contact with particles emitted by pneumatic seed drills during the sowing of maize. From the outset it was desirable to verify that the hypothesis could be applied to the realities of the field, correlating also the environmental facts during the period under examination, such as, for example, the relative humidity of the air. Next, together with Professor Tapparo's team, the particles of seed shell emitted into the atmosphere by the seed drill were analyzed using PM and PM10 detectors. Specifically studied, were the quantity, as well as the dimension of the cloud of powder which was produced. These parameters

proved to be fundamental in determining the size of the “toxic cloud” which formed around the operating seed drill, with the consequent probability that bees were lethally poisoned. To assess the toxic cloud a method of exposing bees to it was developed. These were called “mobile cages” and allowed the simulation of a single flight of a foraging bee over a plot during sowing, controlling the height and time of exposure of the bee to the particles. Still in collaboration with the Department of Chemistry of the University of Padua, a new method of analysis was put in place to calculate the content of insecticide, in particular neonicotinoids, of samples consisting of single bees. This method is innovative since it allows a precise and rapid description of the phenomena which, up to then had required samples of some thousands of bees, which could produce false results. Furthermore, the method permits, the quantification of insecticide in a single insect, addressing the cause of contamination and exposing the polluters without recourse to large scale trials, and to precisely pinpoint the problem. This study was developed and financed in part under the auspices of the ministerial project Apenet (http://www.reterurale.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/4094).

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CHAPTER I

Translocation of Neonicotinoid Insecticides From Coated Seeds to Seedling Guttation Drops: A Novel Way of Intoxication for Bees

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I collected all the data I report, partially analyzed them and drafted the manuscript

Ecotoxicology

Translocation of Neonicotinoid Insecticides From Coated Seeds to Seedling Guttation Drops: A Novel Way of Intoxication for Bees

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ABSTRACT The death of honey bees, *Apis mellifera* L., and the consequent colony collapse disorder causes major losses in agriculture and plant pollination worldwide. The phenomenon showed in- creasing rates in the past years, although its causes are still awaiting a clear answer. Although neonicotinoid systemic insecticides used for seed coating of agricultural crops were suspected as possible reason, studies so far have not shown the existence of unquestionable sources capable of delivering directly intoxicating doses in the fields. Guttation is a natural plant phenomenon causing the excretion of xylem fluid at leaf margins. Here, we show that leaf guttation drops of all the corn plants germinated from neonicotinoid-coated seeds contained amounts of insecticide constantly higher than 10 mg/l, with maxima up to 100 mg/l for thiamethoxam and clothianidin, and up to 200 mg/l for imidacloprid. The concentration of neonicotinoids in guttation drops can be near those of active ingredients commonly applied in field sprays for pest control, or even higher. When bees consume guttation drops, collected from plant grow from neonicotinoid coated seeds, they encounter death within few minutes.

KEY WORDS: guttation, neonicotinoid, honeybee, seed coating

Introduction

Phytophagous insects occurring in soil at sowing time tend to concentrate around on corn, *Zea mays L.*, seedlings causing extensive damage. Granular insecticides applied to the soil have been the method of choice for their control for a long time. More recently, the strategy has been surpassed by the seed coating technique using neonicotinoids, which are active against a broad range of pest

species,
including wireworms (*Agriotes* spp.) and the rootworm *Diabrotica virgifera virgifera* LeConte (Altmann 2003).

Among the main reasons of success of eonicotinoids is their systemic action. Upon application on the seed surface, the active compound is translocated and distributed throughout the whole plant, conferring a substantial and long-lasting control of insects and protecting young plants also from sucking leafhoppers and aphids (Magalhaes *et al.* 2009), which are potential vectors of plant virus (Maienfisch *et al.* 2001). Nowadays, neonicotinoids are widely used for seed treatment in cotton (*Gossypium* spp.), sugarbeet (*Beta vulgaris L.*), oilseed rape (*Brassica napus L.*) corn (*Zea mays L.*), and other cereals and crops (Elbert *et al.*2008). The reduced load of insecticide per field unit, allowed by confining it on the seed, represents main advantages in environmental terms compared with former products requiring whole-soil or furrow applications. Elbert *et al.* (2008) pointed out impressive figures revealing the turnover toward insecticidal seed treatment. Starting from: a niche level market of €155 million in 1990, mostly represented by carbamates, by 2005 seed coating developed into a €535 million market, with a 77% share for neonicotinoid insecticides. The loss of pollinating bees is a worldwide crisis. In particular it became manifest as colony collapse disorder (CCD), characterized by a sudden disappearance of worker bees that do not return to the hive. Parasitic mites and viruses have come under suspicion, although no clear conclusions could be drawn as concerns these biotic causes. Pesticides have been shown to be more directly involved and in recent years the attention has been focused on neonicotinoids (imidacloprid, clothianidin and thiamethoxam), a class of insecticides among the most widely used worldwide. The effects of neonicotinoids, such as imidacloprid on honey bees (Suchail *et al.* 2000, Maus *et al.* 2003) could be consistent with the symptoms of CCD. However, the blame on neonicotinoids has not yet been conclusive as the amounts detected in nectar and pollen of plants grown from treated seeds were lower than 10 ng/g (10 ppb), whereas higher doses, as 40 g/l (40 ppb) are necessary for abnormal honey bee foraging behavior, 0.5 mg/l (0.5 ppm) for the first missing bees, and 3 mg/l (3 ppm) for 100% of the bees failing to return to a source of sugar offered to them (Yang *et al.* 2008). In Italy, a highly recurring coincidence between corn sowing time and bee death has been noticed

previously (Greatti *et al.* 2003, Greatti *et al.* 2006), leading to the hypothesis that solid coating debris and dust uplifted from sowing machines could fall over nearby vegetation and contaminate wildflowers. Within the same frame of thought, we postulated that a different and hitherto overlooked source of directly lethal doses could be present in the fields, and we took into consideration the hypothesis that intoxicating concentrations could accumulate in guttation drops of young corn plants. Guttation (from the Latin "gutta" drop) is the formation of drops of xylem sap on the tips or along the edges of leaves. It is a physiological phenomenon occurring in many vascular plants, in particular grasses, water entering roots creates a slight pressure that forces it to rise and be exuded through the hydatodes at leaf margins. This is a regular occurrence in many plants and is not restricted to nighttime, although in the dark stomatal closure can lead to higher internal pressure that increases guttation drop volumes, thereby enhancing the visibility of the phenomenon (Goatley and Lewis 1966, Koulman *et al.* 2007). Guttation is common but often unnoticed as easily confused with dew characterized by small condensation drops from atmospheric humidity. Guttation drop can roll off, evaporate or may be sucked back into the leaf (Chen and Chen 2007).

Bees require intense drinking activity (Visscher *et al.* 1996, Kuhnholz and Seeley 1997) and have been reported to collect guttation water (Shawki *et al.* 2005).

In the current study, we wanted to verify whether neonicotinoids used for seed coating could be translocated in guttation drops and reach concentrations toxic to bees. In parallel, we tested the toxicity of serial concentrations of these insecticides by setting up a test apt to evaluate, in reasonably short time, the appearance of intoxication symptoms in bees upon consumption of neonicotinoid aqueous solutions.

Materials and Methods

Insect Origin.

Trials were carried out in the exper- imental farm of the faculty of Agriculture (University of Padova) located in Legnaro, Italy. Bees **(***Apis mellifera* L.) used for the tests were collected from different colonies residing within the farmers field facilities.

When season allowed the bees to fly, they were collected with a net in front of the hive; otherwise (in winter), bees were collected from within the hive.

Insecticides Tested.

Trials started in spring 2008. Guttation drops were collected from corn seedlings germinated from commercial seeds coated with the neonicotinoids imidacloprid (Gaucho 350 FS, Bayer Cropscience; 0.5 mg/seed), clothianidin (Poncho, Bayer Cropscience AG, Leverkusen, Germany; 1.25 mg/seed), thiamethoxam (Cruiser 350 FS, Syngenta International AG, Basel, Switzerland; 1 mg/seed), and fipronil (Regent 500 FS, BASF SE, Ludwigshafen, Germany; 1 mg/seed). The last nonsystemic compound is a member of the phenyl pyrazole class of pesticides. Each of the four insecticides mentioned was a regularly registered product for corn seed coating in Italy in 2008. Seeds used (hybrid PR34N84) were from Pioneer Hi-Bred Italy (Johnston, IA), and all also were coated with the fungicide Celest XL (Syngenta), based on Fludioxonil (2.4%) and Metalaxyl-M (0.93%). The untreated control was also from Pioneer Hi-Bred (for biological agriculture) and belonged to the hybrid PR33A46. In field crops, we had cases treated with each of the above-mentioned compounds. For potted plants, we focused essentially on imidacloprid.

Collection of Guttation Drops.

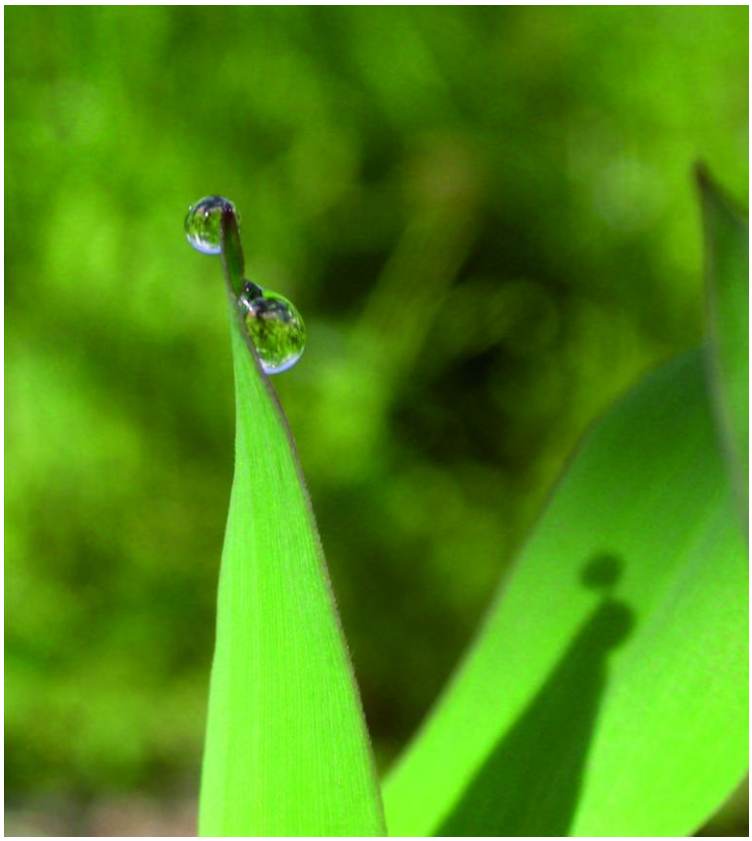
During spring (April) corn seedlings were grown in open field, spaced 20 cm within the row and 75 cm between rows. In subsequent periods (May), tests were replicated by sowing coated seeds in pots with a diameter of 15 cm and growing two to five plants per pot in the laboratory. In total, six to eight pots for each compound were used and equal numbers were sown with uncoated seeds as control, or with seeds coated with fungicides. For each seedling, we gathered all guttation drops at all plant levels, by using Pasteur micropipettes. Collection in the field was carried out from 8:00 to 9:00 a.m. from all plants within a row, until a volume of 5 ml was available. In the laboratory, because guttation occurs throughout the days and night, it was possible to collect them three times a day, yielding a volume of 1-2 ml/d. Samples were stored at 2 C. Half of the volume was sent for chemical analyses and half for toxicity bioassays, which were normally performed within 2-3 d. Collection of guttation drops was carried out from corn emergence up to the first 3 wk for each of the treatments as subsequently the phenomenon ceased in its intensity both in the field

and in the laboratory.

Insecticide Content in Guttation Drops.

Pure chemicals for the preparation of solutions of thiamethoxam, clothianidin, imidacloprid, and fipronil, to be used for reference toxicity curves and as analytical determination standards, were from Fluka (Sigma- Aldrich Group, Milan, Italy; Pestanal, purity 99,7% for thiamethoxam, clothianidin, imidacloprid, 97.6% for fipronil). Methanol (VWR, International, Milan, Italy), and acetonitrile (Riedel de Hae¨ n, Sigma-Al- drich Group) were of high-performance liquid chromatography (HPLC) grade. Pure water was produced by Milli-Q equipment (Millipore, Billerica, MA). HPLC analytical determinations were performed on a 680 chromatography system (Dionex Corporation, Sunnyvale, CA) equipped with UV-Vis diode array detector, a 20- l sampling loop of the injector valve and an Alltech Alltima C18 analytical column (5 m, 4.6 250 mm; Altech Associates, DeerÞeld, IL),

Fig. 1. Guttation drops on corn leaves in the field. (Online figure in color.)



according to published methods (Ying and Kookana 2004, Singh *et al.* 2004, Rancan *et al.* 2006, Zhou *et al.* 2006) optimized for different matrices. The following instrumental procedure was optimized: eluent flow rate of 1.2 ml/min, gradient elution (0 - 4 min, 70:30% water/acetonitrile; 4-9 min, linear gradient to 100% acetonitrile; 9-13 min, 100% acetonitrile), 20 C of column temperature, multiwavelength acquisition of detector signal and analytes quantification at 252 nm for thiamethoxam, 269 nm for clothianidin and imidacloprid, and 215 nm for fipronil. Instrumental calibration (external) was performed by analysis of standard solutions in the 0.1-100 mg/liter concentration range of analytes in methanol. The analysis of guttation solutions was performed by direct injection, after filtration on a Millex HV 0.45-m syringe filter (diam. 4 mm; Millipore) of 100 -300 l of the sample.

Toxicity of Guttation Drops to Honey Bees.

The test was carried out at 20 -22 C in a temperature controlled room. Before the tests bees were kept in cages (20 by 20 cm) for at most 2 h without water and food. Single bees were sampled from the cage with test tubes and introduced into 5-cm-sided cubic net (tulle) cages. After 15 min of adaptation, the guttation water was offered. We tested either plain guttation drops or guttation drops with the addition of 15% honey (vol: vol) (21%, vol:wt, according to specific weight of honey of 1.4). For bees to be attracted to drink, 30 µl was placed on the top of the net cage inside a capillary glass tube (100 mm in length with a diameter of 1 mm).Actual liquid consumption was ascertained by variation of the level in the capillary, and a drinking event was defined by the consumption of a minimum of 5 µl of liquid (bees that did not accept to drink within 5 min were discarded). After 20 min from solution consumption, a drop of honey was offered on the top of the cage to feed bees. The bee was constantly observed and from the first event of drinking, that normally occurred shortly after offering the capillary with solution, we recorded the time required for the appearance of two intoxication symptoms that always occurred before death. The first was a jerky inward arching of the abdomen, and the second was a definitive block of the flight capability caused by a paralysis of the thorax muscle and therefore of the wings.

Evaluation of Dose–Response Effect.

To observe the relationship between concentration and response of the above mentioned two intoxication symptoms, we offered bees with solutions of pure insecticides in water with 15% honey, at increasing insecticide concentrations using the same method described above for guttation drops. We started with concentrations of 100 mg/liter with progressive halving, up to dilutions no longer

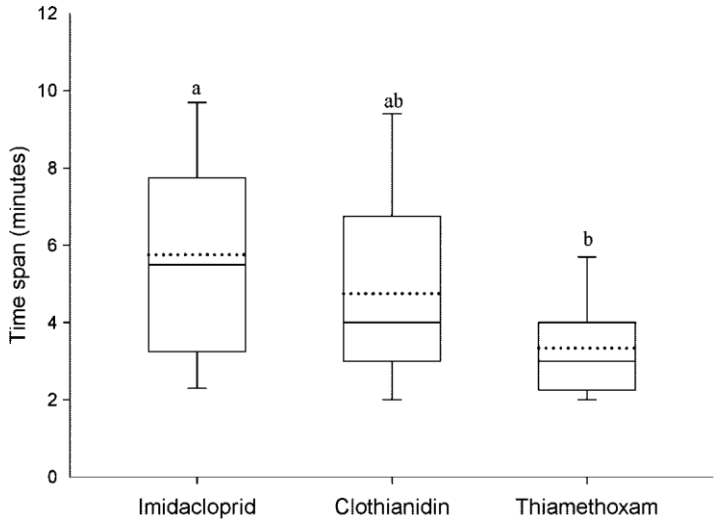


Fig. 2. Time between appearance irreversible wing-block and drinking of guttation drops collected on leaves of field corn crops, from three marketed neonicotinoid-coated. Guttation sampled on plants germinated from untreated seeds did not show any toxicity. The whisker represents the maximum and the minimum of the recorded time; the dotted line indicates the average; the upper, middle, and lower lines of the box indicate the 75, 50, and 25% of the time, respectively. Bars marked with different letters indicate significant differences ($P < 0.05$; Tukey-Kramer test).

causing, within 1 h, the two intoxication symptoms in all bees which had drink at least 5µl. We tested each dosage, for each of the three neonicotinoids (imidacloprid, clothianidin and thiamethoxam). We also assayed a saturating dose (3.8 mg/l) of the non-neonicotinoid fipronil. Each treatment was repeated on a minimum of 12 bees, separately tested, for each concentration. The actual concentration of insecticide in the solutions, obtained by theoretical dilution, was confirmed by chemical analysis.

Statistical Analysis.

The time between drinking from guttation drops (from three marketed neonicotinoid-coated seeds) and the appearance of intoxication symptom, as well as different concentrations of chemicals in guttation samples were compared by one-way variance analysis (ANOVA). Subsequently, a significance difference test (Tukey-Kramer) was applied.

Results and Discussion

Collection of Guttation Drops.

First, we observed that the totality of corn plants in the field feature abundant and continuous guttation drops during their first 3 week of growth (Fig. 1). Although the guttation water tends to evaporate during the warmer hours of the day, in corn seedlings, guttation drops can flow down into the crown cup and persist drinkable for most of the day. Although textbook definitions tend to relate guttation to conditions of moist soil and humid air, the phenomenon is not restricted by these parameters that only enhance the size of the drops facilitating their observation. Moreover, guttation drops formed under conditions of lower soil moisture and dryer air can contain even more concentrated solutes as a consequence of the progressive water evaporation. During April-May, guttation drops were regularly found on vegetation until 9 -10 a.m. Only on particularly windy days drops were not found. From potted plants in the lab the collected amount was 30-150 l/die per plant, whereas in the field a single collection in the morning easily allowed to gather 1-3 ml from 100 plants.

Insecticide Content in Guttation.

Chemical analyses of the guttation water from laboratory grown corn plants during 3 week of growth showed the presence of the corresponding seed coating neonicotinoids in all samples. Guttation drops collected on plants from neonicotinoid coated seeds contained concentrations of each respective active ingredient of (mean ± SE) 47 ± 9.96 mg/liter for imidacloprid, 23.3 ± 4.2 mg/liter for clothianidin, and 11.9 ± 3.32 mg/liter for thiamethoxam with statistically significant differences (ANOVA: $F = 7.51$; $df = 2, 15$; $P = 0.005$). The amount of imidacloprid found in drops of plants grown from seeds treated with 0.5 mg per seed was significantly more concentrated than that of thiamethoxam in guttation drops of plants treated with 1 mg of active ingredient per seed ($P < 0.01$; Tukey-Kramer test). The nonsystemic fipronil was never found above its detection limit in guttation water.

The higher translocation from seed to guttation observed for imidacloprid is surprising in light of its lower amount in the coating compared with thiamethoxam and clothianidin. In another experimental analysis carried out on drops produced from individually potted plants obtained from seeds coated with imidacloprid average concentrations resulted of 82.8 ± 14.07 mg/liter, with maxima reaching over 110

mg/liter. Therefore, as first approach neonicotinoids concentration on guttation does not seem strictly dependent on density of plants per pot.

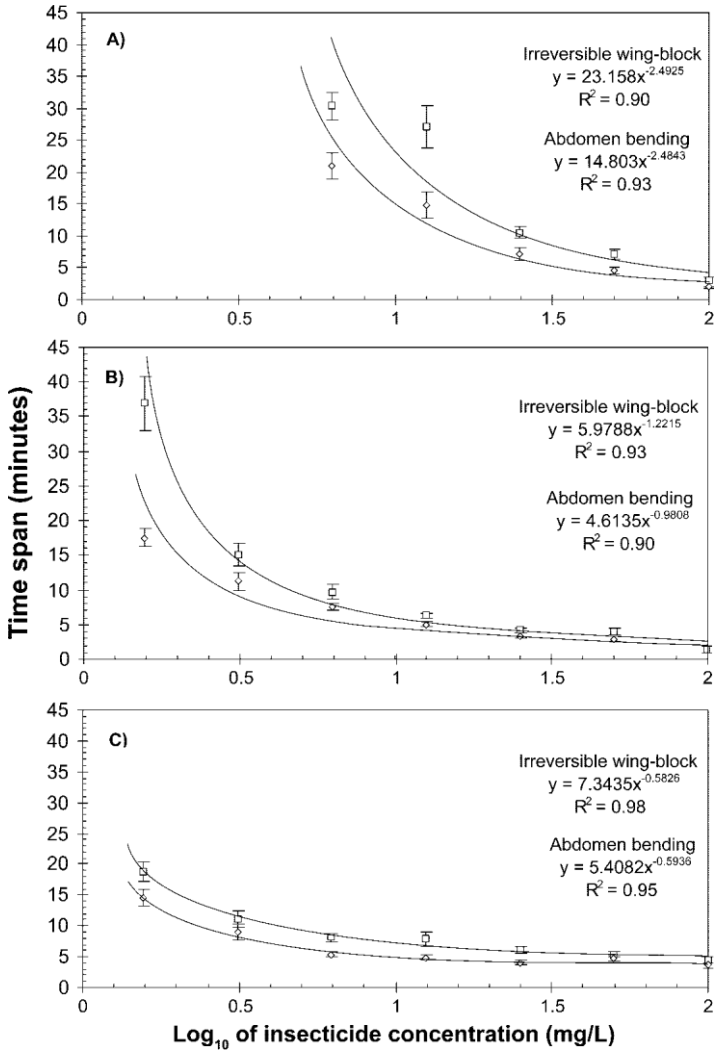


Fig. 3. Toxicity of neonicotinoid imidacloprid (A), clothianidin (B), and thiamethoxam (C) on honeybees. Neonicotinoid toxicity was calibrated as time of appearance after drinking of two poisoning symptoms (squares, irreversible wing-block; diamonds, abdomen bending) upon offering bees drops of water with 15% honey containing insecticides (pure chemical). Each symbol represents the mean of 12 replicates, and the vertical bars indicate the standard error of the means. Concentration data (milligrams per liter) are transformed in $\log_{10}$.

Neonicotinoid concentration in guttation drops resulted in general rather variable presumably due to environmental factors as concentration via water evaporation, collection time of the day, and time elapsed since seedling emergence. In more recent experimentation for all three neonicotinoids peak concentrations above 100 mg/liter were observed and also 200 mg/liter for imidacloprid. These values are near or even higher than those of insecticides commonly applied in field sprays for pest control. Regardless, insecticide translocation from seed and accumulation in guttation seems rather clear and efficient.

Toxicity of Guttation Drops to Honey Bees

After consumption of the toxic drops, the first noticeable effect was a generic agitation similar to that occurring upon starvation. The first objective intoxication symptom was an arching of the abdomen (probably a stinging reflex). At this stage, the insect still retains its flying capability and profuse regurgitation events can often be seen. Subsequently, the bee undergoes wing paralysis and uncoordinated movements. The latter event constantly resulted an irreversible stage for all the tested guttation, thus constituting an objectively recordable cue of the subsequent lethality. Death, as defined by complete stillness, was not plotted because the time between wing block and possible residual capability to move a leg resulted extremely variable, as reported by Suchail *et al.* (2000). The test also enabled us to ascertain whether single bees does actually take up the solution offered and at which volume with good approximation.

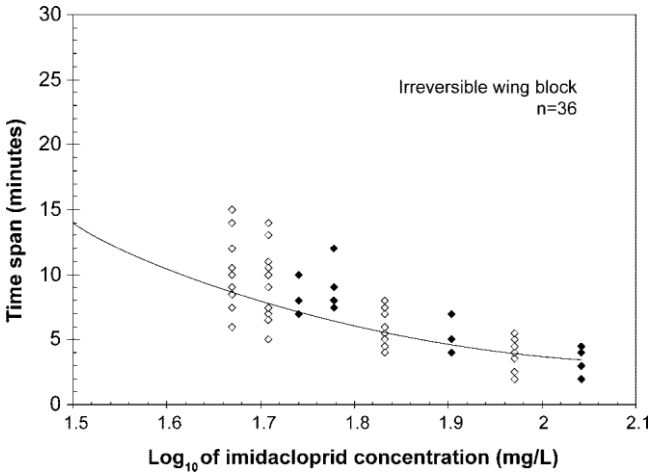


Fig. 4. Time interval between appearance irreversible wing-block of single caged bees and ingestion of guttation drops collected from leaf of potted (10-d-old) corn seedlings from imidacloprid-coated seeds. Concentration was determined by HPLC analysis. The curve corresponds to that shown in Fig. 3A for pure imidacloprid at the higher doses. Black symbols, pure guttation; white symbols, guttation with 15% honey. Concentration data (milligrams per liter) are transformed in $\log_{10}$.

Guttation drops collected in the field on plants grown from commercial seeds coated with the three neonicotinoids considered, offered to bees without honey, caused wing block in a time ranging between 2 and 9 min from consumption (Fig. 2). Those from plants whose seeds were coated with thiamethoxam resulted significantly more toxic in comparison with the imidacloprid coated seeds (ANOVA: $F = 3.71$; $df = 2, 33$; $P = 0.035$). Control guttation drops from noncoated seeds or coated with fungicides did not display toxicity. Guttation drops from plants whose seeds were coated with the nonsystemic insecticide fipronil resulted less toxic or not consistently lethal (data not shown). Thirsty bees consumed immediately the field-collected drops offered in the

cage top but often bees need long periods before drinking causing delays in the test. For such reason, for guttation drops collected from potted plants we added 15% honey to the drops to promptly attract bees to drink. This honey concentration was the minimum ensuring solution uptake within 5 min, a time compatible with the efficiency of the test.

In agreement with data from field collected guttation drops, toxicity of guttation from potted plants germinated in the laboratory from neonicotinoid coated seeds, irrespective to 15% honey addition, showed a strict correlation between active compound concentration and toxicity. In particular for imidacloprid the totality of bees (n 63) that ingested guttation fluid underwent irreversible wing paralysis within a few minutes. Both with pure guttation drops and with those with 15% honey, the wing block symptom was in a range of 2- 4 min for concentrations above 100 mg/liters and of 6 -15 min for those around 50 mg/liters. Guttation toxicity seems clearly related to the neonicotinoid content.

Preliminary tests carried out by offering bees guttation drops of plants from clothianidin or thiamethoxam-coated seeds showed that wing block occurs within shorter times compared with imidacloprid at corresponding concentrations (data not shown). This would con firm that clothianidin and thiamethoxam are more toxic than imidacloprid, although less concentrated in guttation drops, as indicated above. Also, in potted plants, guttation drops from control, untreated seeds plants were harmless to bees.

Evaluation of Dose–Response Effect.

The test devised to verify whether insecticides in water solution with 15% honey could kill drinking bees in short time lapses was satisfactory and of simple setup. Few minutes after drinking from neonicotinoid solutions in lethal concentrations, an excited behavior was observed followed by abdomen bending and wing paralysis as observed for guttation. The two symptoms resulted irreversible for all the neonicotinoid under study at all dosages reported (Fig. 3).

Bees showed a different response to the three neonicotinoids. For clothianidin and thiamethoxam, at the lowest concentrations of 1.5 mg/liter (log10 = 0.18), the chosen symptoms (abdomen bending and wing paralysis) manifested before 1 h. For imidacloprid, the same could be observed at concentrations 6.25 mg/ liter (log10 = 0.8), indicating a lower toxicity toward bees (Fig. 3). Increasing the dosage, the interval between abdomen bending and wing block decreased progressively, becoming nearly

null at 100 mg/liter (log10 = 2) for all neonicotinoids tested (Fig. 3). When using doses lower than the doses reported (Fig.3), either the symptoms did not occur or they did sometimes in reversible manner and in a time exceeding 1 h. Those bees, when fed, would normally survive for at least 24 h. It must be noticed that, as it makes use of a single event of uptake, the test is less severe than those in use to evaluate the median lethal concentration (LC50), for which poisoning solutions are kept available for longer time. Results are in agreement with Yang *et al.* (2008) who reported that the imidacloprid concentration 3 mg/liter in a sugar solution is the threshold preventing bees to return to foraging. This value is close to the one (6 mg/liter) at which we observe a wing paralysis on all insects tested in 1 h. Within each given neonicotinoid concentration, no clear relationship between the actual intake volume and time of appearance of the symptoms was noticed, presumably due to individual response variability and to the frequent regurgitation events that can bias the dose response dependency.

No evident neonicotinoid repellency could be noticed as their concentration neither clearly deter bees from drinking, nor directly affected the volume ingested. These aspects would be the object of future studies.

The effects of pure insecticide solutions (Fig. 3) and those of guttation drops in which a corresponding concentrations was ascertained by chemical analyses, resulted in good agreement. In particular, for imidacloprid the time of appearance of the flight stop distributes with good correspondence along the curve independently obtained by tests in which pure imidacloprid serial dilutions at known concentrations were offered to bees (Fig. 4).

Therefore, the neonicotinoid content in guttation drops seems to satisfactorily explain their toxicity. No additional synergic effect of other compounds present in guttation drops seems to apply in the observed phenomena.

The presence of guttation drops on corn leaves in agricultural crops is easily observable from emergence until up to 3 wk. In northern Italy, this is normally coincident with times from the second week of April to mid-May. Water fetching activity can be rather intensive also in spring, bees are often seen accessing water from different sources and when ground puddles are not available, plant guttation drops represent an exploited alternative. Although, as the season un-folds, blossoming flowers can provide water containing nectar fluids, in early periods bees cannot yet rely on these. It is to be remarked in this respect that in the past 10 yr (in which an outbreak of bee mortality has

been recorded) new cold-resistant corn hybrids have been massively introduced in agriculture that allow an anticipated mid-March sowing in a time that precedes the opening of the majority of wildflowers.

Being the likelihood that bees could drink from corn field or other crops guttation drops not yet quantified, it is still not possible to draw a judgment on a possible correlation between neonicotinoid translocation into guttation drops and CCD. Regardless, the presence of a source of water carrying in solution neonicotinoid concentrations up to the levels shown in the current study, and persisting for weeks on more than a million hectares in the sole northern Italy, is a threatening scenario that does not comply with an ecologically acceptable situation.

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CHAPTER II

Rapid analysis of neonicotinoid insecticides in guttation drops of corn seedlings obtained from coated seeds

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I collected all the data I report, partially analyzed them and in part drafted the manuscript

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Rapid analysis of neonicotinoid insecticides in guttation drops of corn seedlings obtained from coated seeds

Journal of Environmental Monitoring

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Abstract

Regarding the hypothesis that neonicotinoid insecticides used for seed coating of agricultural crops – mainly corn, sunflower and seed rape - are related to the extensive death of honey bees, the phenomenon of corn seedling guttation has been recently considered as a possible route of exposure of bees to these systemic insecticides. In the present study, guttation drops of corn plants obtained from commercial seeds coated with thiamethoxam, clothianidin, imidacloprid and fipronil have been analyzed by an optimized fast UHPLC-DAD procedure showing excellent detection limits and accuracy, both adequate for the purpose. The young plants grown both in pots – in greenhouse – and in open field from coated seeds, produced guttation solutions containing high levels of the neonicotinoid insecticides (up to 346 mg L-l for imidacloprid, l02 mg L-l for clothianidin and l46 mg L-l for thiamethoxam). These concentration levels may represent lethal doses for bees that use guttation drops as a source of water. The neonicotinoid concentrations in guttation drops progressively decrease during the first l0–l5 days after the emergence of the plant from the soil. Otherwise fipronil, which is a non- systemic phenylpyrazole insecticide, was never detected into guttation drops. Current results confirm that the physiological fluids of the corn plant can effectively transfer neonicotinoid insecticides from the seed onto the surface of the leaves, where guttation

Environmental impact

The significant contamination of the guttation drops produced by young corn plants grown from seeds coated with neonicotinoid insecticides may represent a risk for honey bees and other insects. With the aim to assess this possible exposure route for bees, starting from quantitative data, a simple and rapid analytical method for the accurate determination of neonicotinoid insecticides in guttation solutions has been optimized and then applied to different series of real samples collected both in the laboratory and in the field. The optimized procedure could be a very useful tool for the future exposure studies and the consequent risk assessment for honey bees.

drops may expose bees and other insects to elevated doses of neurotoxic insecticides.

Introduction

Honey bee colony losses are a complex phenomenon often characterized by a rapid disappearance of honey bee colonies failing to return to their hive, and the presence of capped brood with a live queen bee and of food stores in the hive, called Colony Collapse Disorder (CCD) syndrome.[1,2] This phenomenon has been observed worldwide in the last few years,[3–5] with a rapidly increasing number of cases in Europe,[6] USA7 and Japan.[3] For instance, over winter 2007–2008, 36% (2.4 million) of America's bee hives were lost.[8] European figures follow the same trend,[6] with peaks of up to 60% of the hives. This honey bee crisis and the consequent reduction in the pollination of flowering plants, induces adverse effects on beekeeping, agriculture and natural ecosystems, and it actually constitutes a worldwide emergency both from an economic and an ecological standpoint.

Many hypotheses, such as infections of parasitic mites,[9] viruses,[10] chronic exposure to sub-lethal doses of insecticides[11–14] or acute effects of neonicotinoid insecticides[15] were formulated to account for bee decline. Up to the present none of them have been confirmed or refuted and their impact has never been clearly quantified, so that a multifactorial origin of colony losses is often suggested in the qualified literature.[3] Moreover, first reports of the surveillance networks on bee decline[6–8] seem to indicate a high temporal and geographical variability in colony losses. In southern Europe significant peak events – different from the winter colony losses – were detected at the beginning of spring.[16,17,6] This supports the hypothesis that they were related to the acute toxic effects of neonicotinoid insecticides released in the environment by agricultural practices, in particular during corn sowing.[16,18] It is worthwhile to notice that in Italy the use of corn seed coated with neonicotinoids was banned in September 2008 and no cases of colony collapse were recorded in the springs of 2009 and 2010.[19,20]

Actually, neonicotinoid insecticides are widely used in agri- culture and the seed coating is used all over the world to ensure a broad range pest control in several crops, including corn (Zea mays L.).[18] Neonicotinoids are water soluble compounds and systemically translocate to plant tissues protecting young plants from root-eating insects and, after emergence, also from sucking insects – such as leafhoppers and aphids – responsible for the transmission of plant viruses.[18] Nevertheless, the neonicotinoids hypothesis of bee decline runs counter to the experimental observation that the amounts of neonicotinoids detected in nectar or

pollen (or dew) of the plants were always lower than 10 ppb,[21] while higher concentrations (>40 ppb) are necessary for abnormal honeybee foraging behaviour or bee loss (>0.5 ppm).[12] Although this prompted investigations into other mechanisms of toxicity for bees, such as the possible effects of sub-lethal doses of insecticides on the course of common bee pathologies, studies on the real ways in which bees are exposed to neonicotinoid insecticides seem to have lacked in quantitative data, so far.

Recently, a novel way of possible exposure (and intoxication) of honey bees to neonicotinoid insecticides was proposed by Girolami and co-workers,[22] who postulated and evidenced the translocation of a significant amount of toxic neonicotinoid insecticide from the coated seed to the guttation drops of young corn plants. Guttation is a physiological phenomenon (often confused with dew) characterized by the exudation of drops of xylem sap through the hydathodes, the porous tissues present at the leaf tops and margins, as an effect of roots pressure.[23–25]

In corn crops, drinkable guttation solutions can persist into the crown cup of the young plants for the whole day.In this work, the effective contamination of the guttation drops obtained from young corn plants grown from seeds coated with neonicotinoids has been studied. With the aim to assess this possible exposure route for bees, starting from quantitative data,[26] a fast liquid chromatographic procedure for the rapid, sensitive and accurate analysis of neonicotinoids in guttation drops has been optimized and then applied to different series of guttation solutions collected both in the laboratory and in the field.

Experimental section

Corn seedlings were obtained from seeds (hybrid PR34N84, Pioneer Hi-Bred Italy) commercially available in 2008, 2009 and 2010 and coated with neonicotinoid insecticides: imidacloprid (N-[1-[(6-chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl] nitramide; Gaucho , Bayer Cropscience, 0.5, 1 or 1.25 mg/seed); clothianidin ((E)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl- 2-nitroguanidine; Poncho , Bayer Cropscience, 1.25 mg/seed); thiamethoxam (3-[(2-chloro-1,3-thiazol-5-yl)methyl]-5-methyl- N-nitro-1,3,5-oxadiazinan-4-imine; Cruiser , Syngenta Interna- tional, 0.6 or 1 mg/seed). Seeds coated with fipronil (5-amino-1- [2,6-dichloro-4-(trifluoromethyl) phenyl]-4-(trifluoromethylsulfi-nyl)-1H-pyrazole-3-carbonitrile; Regent , BASF SE, 0.5, 0.75 or 1 mg/seed), a non-systemic N-phenylpyrazole insecticide, were also utilized. Untreated seeds (hybrid PR33A46, Pioneer Hi-Bred Italy) or seeds coated with fludioxonil and metalxyl-M (Celest , Syngenta

International, 2.4% and 0.93%, respectively) fungicides were used as controls. Corn seedlings were grown both in open field (April 2009 and 2010, seeds spaced 20 cm within the row and 75 cm between rows by using a Monosem NG Plus pneumatic drilling machine) and in the laboratory (greenhouse, November 2008–October 2010) with seeds sown in pots (15 cm in diameter) and growing 2–5 plants per pot. A total of 6–8 pots for each insecticide were used and equal numbers of pots were sown with control seeds (uncoated or coated with fungicides).

For the first 20 days after the emergence of the seedlings, guttation drops were collected every morning by a pipette from the leaves of corn plants (from single plants or homogeneous groups of plants). Samples were stored at 4 °C until the instrumental analysis.

For analytical determinations, a new, fast liquid chromatographic (ultra high performance liquid chromatography, UHPLC) procedure was optimized on a Shimadzu Prominence UFLC-XR chromatograph equipped with a Shimadzu SIL 20AC-XR auto sampler, Shimadzu SPD-M20A UV-Vis diode array detector and a Shimadzu XR-ODS II (2.2 mm, 2 x 100 mm) analytical column with a Phenomenex security guard –Phenom- enex ODS (4 x 2.0 mm) precolumn. The following instrumental parameters were adopted: eluent flow rate of 0.4 mL min-1, gradient elution (0–1 min: 77/23% water–acetonitrile; 1–2.2 min, linear gradient to 100% acetonitrile; 2.2–3.5 min, 100% acetoni- trile), 5 mL of injector volume, 45 ° C of column temperature. Detector signal at l ¼ 215 nm for fipronil, l ¼ 252 nm for thia-methoxam and l ¼ 269 nm for clothianidin and imidacloprid were adopted for analyte quantification. Although thiacloprid and acetamiprid are not used for corn seed coating, they can also be separated and quantified (l ¼ 244 nm) by the optimized analytical method. Instrumental calibration (external) was performed by analysis of standard solutions in the 0.05–10 mg l^{-1} concentration range of analytes in 50% water–methanol. Sample analyses were performed by direct injection of the guttation solutions, after filtration on a Millex HV 0.45 mm (Millipore) syringe filter. Concentrated samples were diluted by addition of a 50% water– methanol solution in the injection vials.

Fipronil, thiamethoxam, clothianidin, imidacloprid, acet- amiprid and thiacloprid were purchased from Fluka (Pestanal, purity >99.7% for the five neonicotinoids and >97.5% for fipronil). Methanol (VWR) and acetonitrile (Riedel de Haen) were of HPLC grade and water was purified by a Millipore MilliQ equipment.

Results and discussion

UHPLC analytical procedure

Trace analysis of neonicotinoid insecticides in environmental matrices is currently performed by conventional reverse phase liquid chromatographic procedures using different detection strategies.[27–32] Even though HPLC-DAD methods are less sensitive and selective with respect to procedures using mass- spectrometric or electro-chemical detectors, our preliminary analysis of guttation drops[22] showed that very high concentration levels of insecticide could be effectively present in these samples. Therefore, the analytical drawbacks typical of ultra- traces environmental analysis (i.e. lack of sensitivity or selectivity in the real samples) could be a minor problem in this case. In other words, the use of a dedicated instrumentation (UHPLC with high efficiency C18 column, 2.2 mm particles) can reduce the analysis time while maintaining high analytical performances, both in terms of sensitivity and selectivity. Actually, the optimized fast procedure reduces analysis time to 5 min (Fig. 1) and no chromatographic interferences have been observed in the detection of the six insecticides in real samples. Precision levels of 0.2% for thiamethoxam, 0.3% for clothianidin and imidacloprid have been computed from replicate analysis of real samples (conc > 2 mg l^{-1}) and 0.8% for fipronil from replicate analyses of standard solutions. The developed method reaches instrumental detection limits of 4.5 mg l^{-1} for thiamethoxam and thiacloprid, 5.1 mg l^{-1} for clothianidin and fipronil, 4.8 mg l^{-1} for imidacloprid, and 5.4 mg $l^{-1}$for acetamiprid, all evaluated using the procedure suggested by IUPAC.[33,34]This means that quantification limits for the analysis of real samples, evaluated as LOQ ¼ 10 x LOD/3,34 are 15 mg l^{-1} for thiamethoxam and thiacloprid, 17 mg l^{-1} for clothianidin and fipronil, 16 mg l^{-1} for imidacloprid and 18 mg $l^{-1}$for acetamiprid.

The linearity range of instrumental responses was tested with up to 100 mg l^{-1} concentrations of standard solutions, obtaining a linear calibration function ($r2 > 0.999$, $p < 10\text{-}8$) for each analyte.

Spiked samples (guttation solutions from seeds coated with fungicides and added with 0.1–1 mg l^{-1} of thiamethoxam, clothianidin and imidacloprid) showed recovery factors in the range 91–108%.

Moreover, the absence of chromatographic interferences for the UHPLC-DAD method was verified by LC- ESI/MS analysis of both spiked and real samples, using identical chromatographic conditions, and obtaining MS signals attributable to the single analyte for each insecticide.

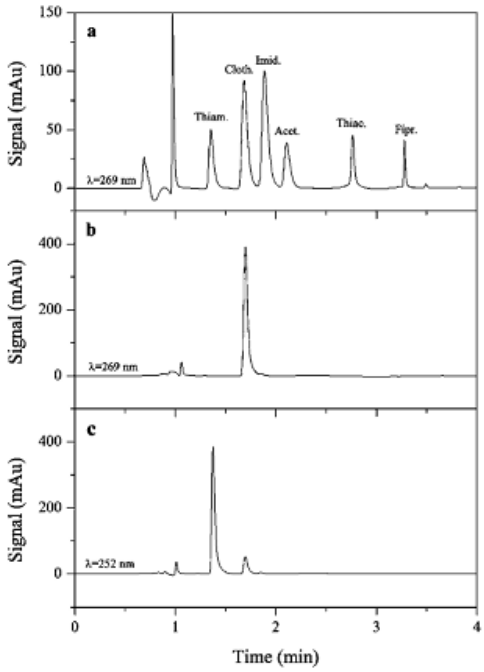

Fig. 1 Chromatograms of (a) 10 mg l^{-1} standard solution of the six insecticides, (b) guttation sample collected from corn plants treated with Poncho® (clothianidin, 42 mg l^{-1}) and (c) guttation sample collected from corn plants treated with Cruiser® (thiamethoxam, 43 mg l^{-1}).

Corn plant guttations

The guttation phenomenon is affected by a number of factors such as humidity, temperature, growth stage, water stress, root depth and soil water potential. Moreover the insecticide residues in guttation fluid exhibit wide variability due both to factors affecting guttation as a phenomenon and to formulation, metabolism within the plant, application methods, adjuvant, solubility of the active ingredient and plant species.[26] Thus, detailed studies need to be conducted to better understand guttation as a possible exposure route to neonicotinoids for honey bees. In this respect, the fast analytical methods described in this paper could turn out to be very useful. Some applications of the proposed procedure are here presented and discussed.

In a first campaign (November 2008) corn plants were grown in pots in greenhouse. The guttation drops collected were divided into six periods in order to obtain enough sample to perform both an UHPLC analysis and toxicological tests.[22] The results of instrumental analysis revealed the effective translocation of the insecticides from the seeds to the leaves of the plants in the whole period when guttation occurs, i.e. 15–20 days after the seedling emergence and with a production of about 30–150 ml/day/plant of water. The concentrations of the insecticides in the guttation drops were surprisingly high for all the three

neonicotinoids while for fipronil, a non-systemic phenylpyrazole insecticide, they were always below the detection limit (LOD ¼ 5.1 mg l^{-1}). Guttation solutions from control seedlings (obtained both in laboratory and in the field from non-coated seeds or from seeds coated with fungicides) contained no detectable concentration of insecticides (e.g., below the instrumental detection limits: 4.5 mg l^{-1} for thiamethoxam, 5.1 mg l^{-1} for clothianidin and 4.8 mg l^{-1} for imidacloprid).

Insecticide concentrations showed a characteristic temporal variation: concentration rapidly decreased during the first 10 days after the seedling emergence (Fig. 2) while it increased again, in the reported experimentation, during the last 10 days of the guttation phenomenon, when it is considerably reduced and water evaporation may significantly concentrate the solute. Thiamethoxam (Cruiser® 1 mg/seed) observed concentration decreased from 24.29 mg l^{-1} during the 1st day after the seedling emergence to 3.55 mg l^{-1} for the 8th–10th days and it increased again to 8.32 mg l^{-1} during the subsequent 10 days. Clothianidin (Poncho® 1.25 mg/seed) concentration ranged from 35.99 mg l^{-1} during the 1st day after the seedling emergence to 8.82 mg l^{-1} for

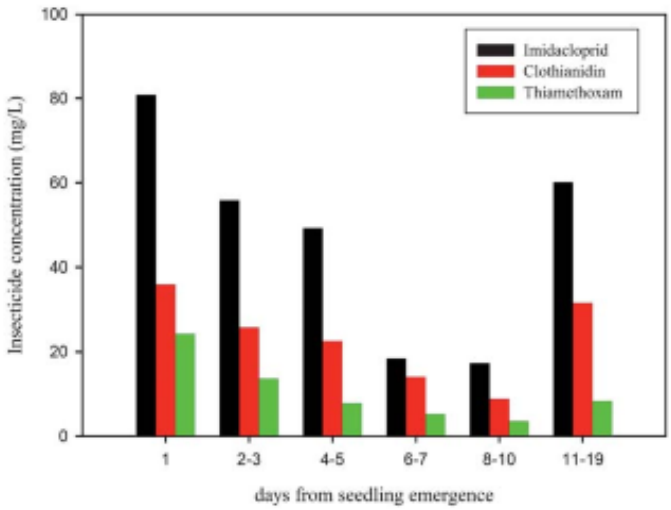

Fig. 2 Concentration of neonicotinoid insecticides in guttation drops of corn seedlings obtained from coated seeds (greenhouse).

the 8th–10th days and it increased again to 31.64 mg l^{-1} during the last 10 days. Imidacloprid (Gaucho® 0.5 mg/seed) concen- tration ranged from 80.87 mg l^{-1} during the 1st day after the seedling emergence to 17.30 mg $^{l-1}$ for the 8th–10th days and it increased again to 60.13 mg l^{-1} during the last 10 days.

Although successive sowing experiments (greenhouse, spring– summer 2009) have always confirmed this temporal trend, a high variability in the translocation efficiency of each

insecticide has been observed (concentrations range up to 346 mg l^{-1} for imidacloprid, 102 mg l^{-1} for clothianidin and 146 mg l^{-1} for thiamethoxam).

Table 1 Concentration ranges of neonicotinoid insecticides in guttation drops collected at the top and at the crown cup of the corn leaves during the first six days after the emergence of the corn seedlings

		Insecticide concentration (mg L^{-1}) in guttation drops	
Corn seed	Active principle	At the top of the leaves	At the crown cup
Gaucho®, 1.25 mg/seed	Imidacloprid	345.8–102.9	120.4–8.2
Poncho®, 1.25 mg/seed	Clothianidin	101.7–76.2	47.0–7.3
Cruiser®, 1 mg/seed	Thiamethoxam	40.8–16.2	25.5–2.9

It is worthwhile to note that, from our results, the insecticide concentrations in guttation drops seem to be only partially related to the original amount in the seed: for instance, the growing of single seedlings per pot produced guttation drops, in the first six days from the emergence, containing decreasing concentrations of imidacloprid, in the range 115–25 mg l^{-1} from seed treated with 1.25 mg/seed and 110–64 mg l^{-1} from seed treated with 0.5 mg/seed. This seems to support the hypothesis that both environmental and physiological conditions (i.e. soil temperature and moisture, air humidity) mainly affect the translocation efficiency and the actual concentration of the insecticides in guttation drops.[26]

In this connection we observed that for seedlings grown under dry conditions (both soil and air), guttations appeared later and a lower volume of water was produced.

On the other hand, under wet conditions the washing-out of the insecticides from the soil is particularly effective for thiamethoxam which is the most water- soluble neonicotinoid. In a trial conducted in experimental parcels (greenhouse, November 2009) using usual soil with three different levels of moisture (obtained by different water supplies), we observed concentrations of thiamethoxam in guttation drops in the range 14–155 mg l-1 in plants grown under wet conditions (the water content in the soil was near saturation), † 27–253 mg l-1 with moderate soil humidity (the parcel had a water content approximately close to the field capacity (FC)) and 34–1154 mg l-1 under dry conditions (the parcel had a water content slightly above the wilting point (PWP).

The comparison between guttation drops collected from the top and from the crown cup of the leaves evidenced that significantly lower concentrations of the insecticides are present in

† It is possible to define as saturated a soil with all pores filled with water. After 24–48 h, when free drainage occurs, the soil reaches the field capacity (FC). When the plants have extracted all water present in the soil they can, the permanent wilting point (PWP) condition is obtained.[35]

the latter (Table 1). This is probably due to the dilution of guttations by dew or to degradation processes of the insecticides, for example photodegradation.

In open field cultivation, both the high contents of neonicotinoids in guttation drops and the characteristic exponential decay of the concentration during the first 10 days after the emergence were confirmed,[26,36] but with higher concentration variability than that observed in greenhouse. For instance, the parallel field cultivation (April 2010) of different coated seeds produced guttation drops with concentration peaks (1st day after the seedling emergence) in the range 77–222 mg l^{-1} for imidacloprid, 19–46 mg l^{-1} for clothianidin and 79–227 mg l^{-1} for thiamethoxam.

We also observed that guttation samples often contain traces of other neonicotinoids than the seed coating insecticide. This is possibly attributable to a contamination effect during the coating procedure, as confirmed by an analysis of the original seeds, during which we found 30 mg/seed of thiamethoxam in 2008 Gaucho® seeds (1.25 mg/seed of imidacloprid). Nevertheless, all guttations from plants grown from Cruiser coated seeds (thiamethoxam) contain correlated concentrations of clothianidin (ca. 10% with respect to the coating insecticide, Fig. 1c) which is a well-known degradation product of thiamethoxam.[37]

As for the toxic effects of these guttation solutions if orally administered to honeybees they induce two characteristics neurotoxic symptoms, i.e. abdomen contraction and irreversible wing block. The time scale is of a few minutes and the concentration of the neonicotinoid insecticides was so high that all the honeybees tested died in up to fifteen minutes.[22] As the time scale is so short, guttation drops could explain the sudden disappearance of worker bees during the early spring if they use corn guttations for their foraging. Literature[23] and direct beekeepers' observations report that guttation drops can be used by honeybees for their foraging especially in the early spring when they require intensive drinking activity and water- fetching for the hive.[24]

However, honeybees are likely to use guttation for their foraging in particular conditions of drought when no other major visible sources of water are present thereabout.

Conclusions

A fast UHPLC-DAD analytical procedure has been optimized for the rapid determination of neonicotinoid insecticides in guttation drops. The method reduces the analysis time to 5 min and shows adequate sensitivity, selectivity and excellent repeat- ability and detection limits for the intended purpose. The method has been successfully applied to the analysis of real

samples obtained from corn seedlings grown both in greenhouse and in open field, confirming the effective translocation of neon- icotinoids from coated seeds to seedling guttations. These solutions may represent a possible route of exposure to lethal doses of the insecticides for bees and other insects.

Because guttation is affected by several factors that cause a high variability both in its intensity and in the insecticide content, further experiments are needed to better understand the phenomenon and the consequent risk assessment for honey bees. The fast analytical procedure described could be a very useful tool for more accurate exposure studies. In any case, the presence of a source of water carrying neonicotinoid concentrations in solution up to the levels shown in the current study, and persisting for weeks on more than a million hectares in northern Italy alone, is a threatening scenario that seems to be incompatible with ecologically acceptable conditions.

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CHAPTER III

Lethal aerial powdering of honey bees with neonicotinoids from fragments of maize seed coat

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Lethal aerial powdering of honey bees with neonicotinoids from fragments of maize seed coat

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Abstract

Losses of bees have been reported in Italy concurrent with the sowing of maize coated with neonicotinoids where pneumatic drilling machine were used. Solid particles with systemic insecticide, falling on the vegetation surrounding the sown area, were thought to poison bees foraging on contaminated nectar and pollen. However, bees fed with guttation drops and dew collected from the surrounding vegetation of sown fields showed no acute toxicity. Chemical analysis showed a relatively low content of neonicotinoid in dew and guttation. Thus, the acute poisoning of bees linked to the vegetation contaminated by seed coated fragments containing neonicotinoids was again unproven. For this reason the direct aerial powdering of bees was investigated exposing caged bees around the sown area, not in contact with vegetation. High or low toxicity emerged in different trials. The synergistic effect on bees of high humidity on toxicity of powder containing neonicotinoid was hypothesized. A clear indication that bees were killed by powdering, only if held in high humidity, emerged. Chemical analysis showed high quantities of neonicotinoid insecticide in dead bees earlier exposed to dust in the field.

Key words: *Apis mellifera*, neonicotinoids, seed coating, toxic powder, humidity influence.

Introduction

In the last decades the European and American honey bee heritage has been subjected to heavy and sudden losses (Potts *et al.*, 2010). In Europe colonies decreased from over 22.5 million in 1990 to about 15.5 million in 2008 (FAO, 2009). The main causes of those deaths are attributable to viruses, fungi (*Nosema* spp.) and to the parasitic bee mite *Varroa destructor* Anderson et Trueman (Thompson *et al.*, 2002; Ribiere *et al.*, 2008;

vanEngelsdorp and Meixner, 2010). Pesticides were also blamed for colony losses (Barnett *et al.*, 2007; Karise, 2007), in particular neonicotinoid insecticides that are widely used for seed coating in crops such as maize (*Zea mays* L.), sunflower (*Helianthus annuus* L.) and winter rape (*Brassica napus* L.). Neonicotinoids are used in the coat to protect the seeds and young plants from wireworms (*Agriotes* spp.), cutworms (*Agrotis* spp.), western corn rootworm *Diabrotica virgifera* (Le Conte) and from numerous species of aphids and leafhoppers (Altmann, 2003). In the last few years sudden losses of foraging bees, with accumulations of dead insects in front of the hives, have been observed during maize sowing period, from mid March to May, in the maize growing regions of Italy and Europe (Bortolotti *et al.*, 2009; Pistorius *et al.*, 2009). The death of bees seems to be correlated with the use of seeds coated with neonicotinoids sown using pneumatic drilling machines (Greatti *et al.*, 2003), but the correlation is not always clear so further studies are required (Giffard and Dupont, 2009). The finding that the pneumatic drilling machine, during the sowing, emits into the atmosphere fragments of seed coat (Greatti *et al.*, 2003), has suggested the hypothesis that dressing fragments containing insecticides falling on the herbaceous vegetation on the margin of the fields, by virtue of the systemic properties of neonicotinoids, penetrate into plants, contaminating nectar and pollen (Greatti *et al.*, 2006). Nevertheless, the amount of insecticides found in vegetation did not seem to justify such rapid losses during, or immediately after the sowing, since the insecticide content is about 50 ppb (Greatti *et al.*, 2006) that is too low a dose to cause poisoning by ingestion according to Yang *et al.* (2008), even if sub-lethal effects over the long period can be considered (Colin *et al.*, 2001; Suchail *et al.*, 2001;Colin *et al.*, 2004; Decourtye *et al.*, 2004; Medrzycki *et al.*, 2003; Maini *et al.*, 2010; Laurino *et al.*, 2011). Chemical analyses of dead bees have also confirmed the presence of neonicotinoids Sabatini *et al.*, 2008) even if the amount of the insecticide did not, as a rule, seem sufficient to induce acute mortality, considering the oral intake LD50 of 40-80 ng/bee (for imidacloprid) reported by Maus *et al.* (2003). Lethal sources of neonicotinoids in the field during maize sowing have been identified but obviously the mechanism by which bee come into contact with them have not yet been. Lethal concentrations of neonicotinoids in the field were found in guttation drops of *Z. mays* (Girolami *et al.*, 2009) but the sudden death phenomena that occurred during the sowing cannot be explained since the guttation appears after plant emergence, at least a week after sowing. This study investigates two hypothetical mechanisms through which honey bee can come into lethal contact with the insecticide used to coat maize seed during the sowing. The first hypothesis is the direct

contamination during sowing, of dews and guttation drops, on the marginal vegetation, by coating fragments containing water-soluble insecticides (before absorption into the plant as previously reported). This was considered as a possible source of poisoning for bees when they collect water for the intensive spring foraging on flowers. The second hypothesis was the possibility that bees could be directly poisoned with the fragments emitted by the drilling machine, that is a possible direct aerial contact of foragers with the dust where there is no contact with the contaminated vegetation. Bee deaths, however, are not regularly observed during maize sowing, so the possibility was considered, that the toxicity of bee dusting could be influenced by particular environmental conditions.

Materials and methods

Experimental sites and insect origin

Field trials took place at the experimental farm of the Agricultural Faculty (University of Padova) located in Legnaro (Veneto Region - 45°20'29.07"N 11°57'30.03"E). The Padova Beekeeping Association (A.P.A. Pad) supplied 7 hives. For the trials, the insects were caught with a net in front of the hives. The bees were kept in tulle mesh cages 20 cm x 20 cm x 20 cm and repeatedly fed with honey drops on the top of cages. Bees inside the larger cage, in sunny days (but not in rainy days), were freed in the evening and renewed daily. At the time of the tests, caged bees were collected (from the 20 cm cage) in a test tube and transferred each one in smaller cubic cages of 5 cm in tulle and again fed with drops of honey placed on the top.

Seed employed

For the trials three batches of seed were used: one of 2008, a second of 2009 and another of 2010 hereafter called "2008/2009/2010 coating" respectively. The 2008 seeds, hybrid PR34N84 from Pioneer Hi-bred Italy (Johnston, IA), were coated with the fungicide Celest® XL (Syngenta; Fludioxonil 2.4% and Metalaxyl-M 0.93%) and insecticide Poncho® (Bayer Cropscience AG., Leverkusen, Germany; Clothianidin 1.25 mg/seed) (Andersch and Schwarz, 2003; Altmann, 2003). For the 2009 and 2010 coating the hybrid employed was X1180D 964890 from Pioneer Hi-bred Italy, coated with Celest® XL, Celest® XL + Poncho® and only for 2009 Celest® XL + Cruiser® 350FS (Syngenta International AG, Basel, Switzerland; Thiamethoxam 1 mg/seed) (Robinson, 2001; Maienfisch *et al.*, 2001). The seeds were supplied in 2009 and 2010 by A.I.S. (Italian seed association) courtesy of MiPAAF (Ministry of Agriculture, Food and Forestry) for

the research project Apenet. The 2009 and 2010 seed batches have a quantity of dust abrasion under the limit of 3 g/q. The quantity was tested with the Heubach test, considered the method which best allows standardization of dust abrasion measurements within the seed industry (Apenet, 2009; Nikolakis *et al.*, 2009). The 2008 batch was a common commercial seed. Drilling machines and sowing A Monosem NG Plus (Monosem, Largeasse-France) pneumatic drilling machine was used for all the sowing operations. Normally 73,000 to 74,000 seeds per hectare were sown (75 cm between rows, 18 cm between seeds in the row). The drill moves at 6-7 km/h with a seeding width of 3 m and, theoretically requires 30 min to sow 1ha. The air waste pipe is situated on the right hand side of the machine and expels air (and dust) at ≈ 150 l/min, at a height of 1.8 m and an angle of 45° to the horizontal. After the experimental employment the machine and the seeding equipment was cleaned with a current of compressed air and, where possible, with water spray. Toxicity of dew and guttation on marginal vegetation In trials 1a, 1b, 2a and 2b (table 1), an area of 3,500 m^2 (70 x 50 m North-South oriented) was sown with seeds treated with both the 2008 and 2009 coating of Clothianidin. In the first instance (trials 1a and 2a) seeds with the 2008 coating were sown and 30 minutes later, seeds with the 2009 coating (trials 1b and 2b) were sown. After the sowing, samples of dew and guttation drops of 5 ml were separately collected from the vegetation on the margins of the sown area, on the East and West side. The first samples were collected before the starting up of the drilling machine as a control, a second at the end of the first sowing (after 30 min) and the third after the second sowing (after 60 min) (for a total of 6 samples, 3 East and 3 West). The day after the trial, repeat samples were collected in the same way (table 1, trial 2a and 2b). In all the trials the drops were collected using a glass Pasteur pipette, put in sealed glass vials and stored in a refrigerator (at 2-4°C).

Table 1. Details of field trials carried out to evaluate the toxicity of dew and guttation.

No.	Date	Starting time - length (min)	Insecticide and coating year	Meteorological conditions				No. bees tested
				T (°C)	RH (%)	wind direction	wind speed (m/s)	
1a	13/V/09	9.00-30	Clothianidin - 2008	20	73	N	2.1	18
1b	13/V/09	9.30-30	Clothianidin - 2009	20	73	N	2.1	18
2a	14/V/09	9.00-30	Clothianidin - 2008	21	79	ENE	2.6	18
2b	14/V/09	9.30-30	Clothianidin - 2009	21	79	ENE	2.6	18

For toxicity test, 15% honey was added to a part of the samples and fed to the bees on the day of collection. Drops of the mixture of 30 µl were placed on the top of the net cage inside a capillary glass tube (Girolami *et al.*, 2009). For each sample at least 6 bees were tested. Samples of dew and guttation on the vegetation of the margin were collected during the trial n. 5b (table 2) of 21/X/2010 (1 h and 24 h after the sowing) for chemical analysis.

Direct field dusting inside cages

The bees were exposed to the dust emitted by the drilling machines for 30 min, inside the small cages (5 × 5 × 5 cm) on the margins of the sowing area and avoiding contact with the vegetation.

Conditions after exposure and influence of relative Humidity

After the exposure to the insecticide dust in the field, honey bees were transferred to a room held at a controlled temperature (22 ± 1.5 °C). For trials where influence of humidity was considered (table 2), half of the cages were kept at the relative humidity of the laboratory lower than 70%, with the use of de-humidifier if needed, hereafter called lab humidity. The other half of the cages were kept at a relative humidity close to saturation (> 95%), hereafter designated as high humidity. To obtain conditions of high humidity, caged bees were held in plastic boxes with Plexiglas sprayed with water on the top and a moistened paper on the bottom. The cages were raised above the paper to prevent the bees getting wet. The humidity was repeatedly checked with an electronic hygrometer and also with a traditional hygrometer (with dry and wet bulb). All the bees were fed with drops of honey on the top of the cages. In trial 2c (table 2), the cages were placed in field on poles at a height of 1.80 m, 20 cages with one bee to a cage were used, 10 were placed on the West side and 10 on the East side of the field. The first was upwind and the second downwind according to the direction of the wind was blowing across N-S orientated plot (table 2). After exposure the cages were taken to the laboratory and held at 22 ± 1.5 °C. Field exposed honey was taken from the top of cages (in which the bees had

died) and was fed to 10 other single caged bees. In trial 3 (table 2), poles were connected by cords and cages were placed around the plot at differing heights (1.5-2-2.5 m). The cages with a single bee inside, were attached to the cord, at intervals of ≈ 2 m; 72 cages were used, 36 on the West side (upwind) and 36 on the East side of the field (downwind). After exposure the cages were taken to the laboratory and held at 22 ± 1.5 °C. Trial 4 (table 2) was similar to the experiment no. 2c. 60 bees were exposed on poles at a height of 1.8 m; 30 cages East side and 30 cages West side of the field. At the end of the sowing (after 30 min) 15 cages of each group were put in laboratory humidity and 15 cages in high humidity. In trial 5a seeds coated with Celest® XL (2010 batch) were sown, for 30 minutes; 60 single caged bees were placed on poles at a height of 1.8 m along both longer sides of the plot. In trial 5b seed treated with Clothianidin (Poncho® -2010 batch) was sown for further 30 minutes and other 60 caged bees were exposed at the same height as trial 4. In trial 5c, during trial 5 b, 60 caged bees were exposed (on poles) not less than 40 meters from the sown area (trial n. 5c). This trial was considered an untreated control.

Chemical analysis

Neonicotinoid content in dew and guttations

Analytical determination standards and analytical methods are reported in Girolami *et al.* 2009) and more specifically in Tapparo *et al.* (2011).

Neonicotinoid content of the maize seed coat

Large fragments taken from the new seed shell coating with Clothianidin (Poncho® Bayer Cropscience AG.- Dormagen – Germany) were collected manually at the air outlet of the drilling machine after sowing experiment. This powder was weighed using an Ohaus AP250D balance (0.01 mg) and dissolved in a known amount of water-methanol (50% v:v) and placed in an ultrasound bath for 20 min. The solution thus obtained, was diluted and filtered using Millex HV 0.45 μm (Millipore) syringe filter and was then analysed by UFLC - DAD procedure, using the method reported below.

Table 2. Details of field trials carried out to evaluate the toxicity on caged bees.

No.	Date	Starting time - length (min)	Insecticide and coating year	Meteorological conditions				No. bees tested	Humidity conditions[3] after exposure
				T (°C)	RH (%)	wind direction	wind speed (m/s)		
2c	14/V/09	9.30-30	Clothianidin - 2009	21	79	ENE	2.6	20	N.C.
3	26/V/09	9.30-30	Clothianidin - 2009	34	34	SE	3.6	72	N.C.
4	10/VI/09	15.00-30	Thiametoxam - 2009	22	41	ENE	2.4	60	L-H
5a	21/X/10[1]	11.00-30	Celest® XL - 2010	18	69	S	2.1	60	L-H
5b	21/X/10*	11.30-30	Clothianidin - 2010	16	71	N	1.9	60	L-H
5c	21/X/10[2]	11.30-30	Clothianidin - 2010	16	71	N	1.9	60	L-H

* Samples of dew and guttation were collected for chemical analysis
[1] Control
[2] Untreated (bees exposed 40 m distance)
[3] L = lab humidity; H = high humidity; N.C. = not specifically controlled

Table 3. Number of dead bees (groups of 6) within 24 h of being fed with water drops collected from the argins of the sowing area, upwind (East) and downwind (West), at different times from the beginning of sowing on 2 consecutive days. Seeds were coated with Clothianidin.

Time from start of sowing	13/V/09		14/V/09	
	Upwind	Downwind	Upwind	Downwind
0 min	0	0	0	1
30 min	1	0	0	0
60 min	0	0	1	0

Neonicotinoid content in bee

Samples of honey bees that died after the sowing of maize coated with neonicotinoid insecticides (Poncho® 1.25 mg/seed, 2010), were taken for analysis of the insecticide content to the Department of Chemical Sciences of the University of Padova. Samples were stored at +2°C for few days before the analysis. The treatment of the samples started with a drying process. The bees were put in a thermostatic oven, at 100 °C for about 2 h. The samples were then ground with a metallic pestle, then put into a solution of methanol- water (50% v:v) and placed in an ultrasonic bath for 20 min. Samples were finally centrifuged, the floatage was separated and filtered with Millex HV 0.45 µm syringe filter (Millipore). The analyses were performed in a UFLC instrument (Ultra Fast Liquid Cromatography, Shimadzu XR -Prominence) equipped with an UV-Vis diode array detectorand a Shimadzu XR - ODS II (2.2 µm, 2 × 100mm) analytical column and a Phenomenex Security Guard pre-column. The following instrumental procedure was optimized:eluent flow rate of 0.4 ml/min, gradient elution (0-0.5min 70:30% water/acetonitrile; 0.5-1.5 min linear gradient to 100% acetonitrile; 1.5-3 min 100% acetonitrile), 45 °C column temperature, multiwavelength acquisition of detector signal and analytes quantification at 269 nm for Clothianidin. Instrumental calibration (external) was performed by the analysis of standard solutions in the 0.1-100 mg/liter concentration

range of analytes, prepared in methanol-water solutions (50% v:v) from pure analytical standards (Sigma-Aldrich Group, Milan, Italy; Pestanal, purity > 99.7%).Methanol (VWR, International, Milan, Italy) and acetonitrile (Riedel de Haen, Sigma-Aldrich Group) were of HPLC grade. Pure water was produced by Milli-Q equipment (Millipore, Billerica, MA).

Statistical analysis

For comparing different mortalities of bees $\chi 2$ test was used.

Results

Toxicity of dew and guttation on the marginal vegetation

In trials 1a, 1b, 2a and 2b (table 1) bees fed with drops collected from the vegetation on the margin of the sowing area did not demonstrate symptoms of poisoning; only 3 bees out of 72 died (table 3) without specific symptoms of neonicotinoid poisoning such as a jerky inward arching of the abdomen (Girolami *et al.*, 2009). There were no subsequent mortalities detected either in the control (0 minutes) before the starting up of the drilling machine, or in other successive samples within 48 h. The bees were then freed, into the sunlight, and almost all were able to fly away. Even the samples collected after two consecutive days of sowing, despite probable higher quantities of fragments of coating on vegetation on the margins, did not demonstrate any acute toxicity to honey bees.

Direct field dusting inside cages

In trial 2c (table 2), bees contained in tulle cages, were exposed to the dust of the drilling machine at the margins of the sowing area for 30 min and then taken to the laboratory. The 10 bees that were exposed West-upwind were all still alive after 24 h, while bees exposed Eastdownwind all died (in the laboratory) (table 4) within 5 to 10 h. To eliminate the doubt that the death of bees could be due to feeding on drops of hypothetically contaminated honey on the top of cages exposed to dust in the field, honey was taken from cages were bees had died and fed to 10 other bees, of which, only one showed symptoms of neonicotinoid poisoning within 24 h. In trial 3 (table 2) bees inside cages were placed around the plot at differing heights (1.5-2-2.5 m). None of the 72 bees taken to the laboratory, after exposure, showed evident symptoms of poisoning within 24 h (table 4). The honey bees exposed in this trial did not die and the height of exposure seems not to be determinant in bee mortality. In trial 4 (table 2) the influence of relative

humidity was evaluated on 2 groups of bees exposed to dust in the field, (30 East side and 30 West side), by holding them in laboratory humidity or in a semi-saturated condition. In high humidity 73% of bees died (22 out of 30).

Table 4. Number of dead bees (2 groups of 10 or 36) exposed on the margins of the sown area to the dust of the drilling machine for 30 min in two experiments.

No. - date of trial	Insecticide	West		East		χ^2*	Probability
		Dead	Survived	Dead	Survived		
No. 2c - 14/V/09	Clothianidin-2009	0	10	10	0	20	$p < 0.0001$
No. 3 - 26/V/09	Clothianidin-2009	0	36	0	36	0	n.s.

* χ^2 was calculated in the same line

Table 5. Number of dead bees (groups of 30) exposed in single cages, to the emissions of the drill, taken to the laboratory and kept in varying conditions of RH.

No. - date of trial	Insecticide	Conditions after exposure	Mortality		χ^2 *	Probability
			dead	survived		
No. 4 - 10/VI/09	Thiamethoxam - 2009	Lab humidity	5	25	19.46	$p < 0.0001$
		High humidity	22	8		
No. 5a - 21/X/10[1]	Celest XL® - 2010	Lab humidity	3	27	-	n.s.
		High humidity	4	26		
No. 5b - 21/X/10	Clothianidin - 2010	Lab humidity	15	15	13.87	$p < 0.0002$
		High humidity	28	2		
No. 5c - 21/X/10[2]	Clothianidin - 2010	Lab humidity	1	30	0.35	n.s.
		High humidity	2	30		

* $\chi 2$ was calculated in the same column and between two humidity conditions
[1] Control
[2] Untreated (bees exposed 40 m distance)

Of the corresponding bees held in lab humidity, respectively only 16% died (5 out of 30), showing highly significant differences in the $\chi 2$ test between the two humidity conditions (table 5). In trial 5a (table 2), only seed treated with fungicides (Celest® XL) was used. Of the 60 bees exposed in the field and then taken to the laboratory, 3 out of 30 died in lab-humidity and 4 out of 30 died in high humidity without significant differences between high and low relative humidity conditions. In trial 5b (table 2), seed treated with Poncho® was used immediately after the sowing with fungicides. High mortality was observed in bees with significant differences between high and low humidity after the exposure. In trial 5c (table 5), bees were exposed at not less than 40 meters from the drilling machine and almost all survived without significant differences between high and low humidity. Therefore, highly significant differences emerged between different humidity regimes when seeds treated with insecticides were used whilst, using seeds treated only with fungicides or holding the cages a distance from the drilling machine, no significant differences emerged. Comparing the mortality between fungicide and insecticide exposed bees (table 5, trials 5a and 5b), separately in high humidity or lab

humidity, highly significant differences emerged (figure 1). Therefore high humidity increases mortality only when insecticide is used and not with fungicide.

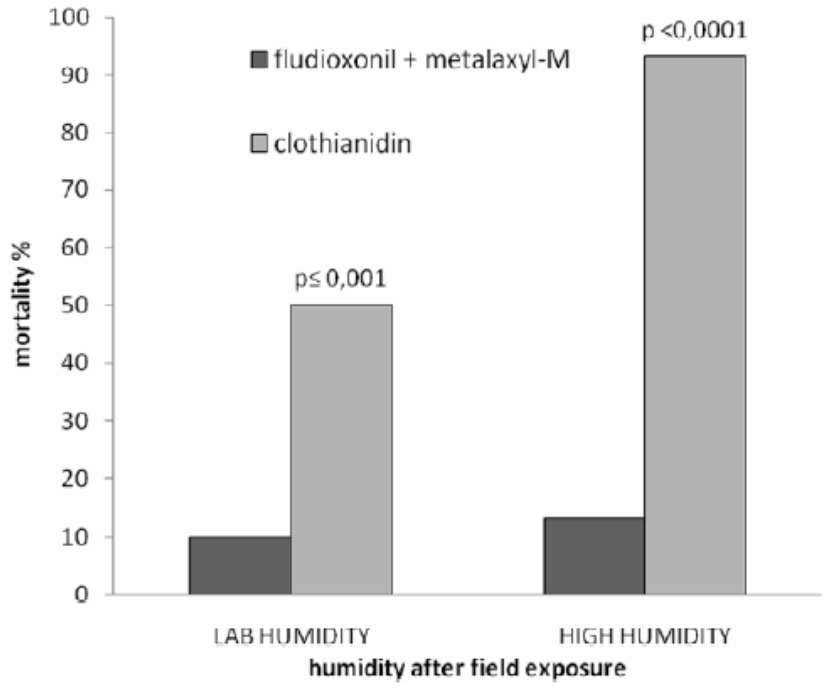

Figure 1. Percent mortality of *A. mellifera* exposed to the dust emissions of the drilling machine using maize treated with only fungicides or fungicides plus insecticide (table 5, trial 5a and 5b). p-values refer to mortalities in the same humidity conditions.

Chemical analysis

Dew and guttation analisis

The chemical analysis of the fragments of seed coating showed approximately, or more than, 20% (wt:wt) of Clothianidin a.i. content. In both samples of dew and guttation drops collected one hour and 24 h after the sowing the insecticide used for seed dressing was found in concentrations lower than 30 ppb, with an overall average of 15.87 ppb (table 6).

Neonicotinoid content in bees

Chemical analysis of dead bees found an average of 279 ± 142 ng/bee of Clothianidin in high humidity while in low humidity the average was 514 ± 174 ng/bee, with an overall mean of 396 ng/bee.

Table 6. Content of Clothianidin in samples of dew and guttation drops collected from vegetation on the field margins after the sowing.

No. - date of trial	Insecticide	Field side	Time of sampling-quantity (ppb)	
			1 h	24 h
No. 5 - 21/X/10	Clothianidin - 2010	East	27	6.5
		West	17.5	12.5

Discussion

Toxicity of drops of dew and guttation

No acute toxicity was found in bees fed with dew and guttation drops collected on the margins of the seeded area even after two consecutive days of sowing in the same plot. Thus the hypothesis that the bees were acutely poisoned by solid fragments falling on the vegetation was again unproven. In particular, it seems that honey bees cannot be lethally poisoned by drinking dew and guttation on vegetation during, or after sowing of maize coated with neonicotinoids. These observations agree with semi-field trials based on the contamination of flowers sprayed with doses of neonicotinoids (imidacloprid) relatively higher than the quantity that would fall during the sowing (Schnier *et al.*, 2003). The absence of mortality is congruent to the neonicotinoid content of dew and guttation drops: chemical analysis showed an average content of Clothianidin of 15.87 ppb. Considering that a bee can drink 30 µl of solution in a single session (Beekman *et al.*, 2004), the intake of active ingredient would be 0.5 ng of Clothianidin. That is a dose more than fifty times lower than that required to cause an acute poisoning with a single ingestion (Girolami *et al.*, 2009). Obviously the absence of acute toxicity of vegetation containing low doses of neonicotinoids cannot exclude other poisoning sources for honey bees that may be present during the sowing with dressed maize. Similarly, the effects of chronic toxicity over a long period, due to sub-lethal doses of neonicotinoids, cannot be excluded (Medrzycki *et al.*, 2003; Aliouane *et al.*, 2009).

Direct field dusting inside cages

The data from the first experiment with caged bees (table 4, trial 2c) implied, as a probable contamination, the direct powdering of bees exposed in small cages, for half an hour to the dust of the drill and unable to fly freely. The hypothesis of direct dusting appeared to be contradicted in trial 3 where no mortality was observed (table 4). The weather conditions between the two trials (table 2) corresponded, the first to spring conditions with a low temperature (21 °C) and high humidity (79%), the second to

summer conditions with a high temperature (33 °C) and low humidity (34%). It was thought that weather variables could influence mortality, in particular it was suggested that given the water solubility of the neonicotinoids, humidity could play a role in the deaths of bees. This hypothesis was tested in a subsequent trial (table 5, trial 4) where exposed bees were kept in the laboratory at different humidity. The mortality of bees kept in high (semi-saturated) humidity was very high, whereas, in lab humidity (≤ 70%), almost all survived (table 5). The influence of high humidity corresponding to weather conditions that frequently are present in spring, in the first few hours of morning sun, was verified in a further trial (table 5, trial 5b). In trial no. 5a, where seeds treated only with fungicides were sown, or bees were kept in cages far from the sown area (trial 5c), a low mortality was recorded in bees held in both humidity conditions. For this reason, it is possible to consider these fungicides coating as not toxic to honey bees, and as an acceptable untreated control. Moreover, in these trials (5a and 5c), no significant differences were found between mortalities in the two humidity conditions, this suggest that high humidity, in itself, could not cause mortality. High humidity, on the other hand, seems to have a synergistic influence on the toxicity of insecticides that come into contact with honey bees. The amount of insecticide found in samples of dead bees (analyzed 24 h after the end of the trial), is sufficient to explain the mortality, because the quantity found are more than 10 times higher (table 7) than the contact LD50 for Clothianidin of 21.8 ng/bee (Iwasa *et al.*, 2004). There are no doubts that the bees tested died because of the high amounts of insecticides that reached them, but the mechanism through which they get contaminated, in particular if the wind has a role, as suggested by the first trial where mortality was observed only downwind, remains to be investigated. From the data reported it is possible to suppose that honey bees die in spring, throughout the maize sowing period, because they are contaminated by insecticide dust emissions during foraging activity when they fly near a working drilling machine. As reported, bees were exposed to the dust emitted by the drilling machine for half an hour without the possibility of flying away, therefore other experimentation to demonstrate that the bees can be dusted in flight, are necessary. The reason why the powder emitted by the drilling machine, independently of the synergistic effects of humidity, had such a dramatic effect on bees may have a rather simple explanation. The fragments expelled during the sowing, contain more than of 20% of neonicotinoid, that is a concentration of insecticide at least 2,600 times greater than that diluted in water for agricultural sprays (for example Dantop®, Clothianidin 50%, is used at 15 g/hl, that is 75 ppm). The presence in the field

of sources of highly concentrated insecticide, sufficient to kill bees, was previously not considered, probably because the lethal effects are contingent upon the differing humidity in the field.

Table 7. Quantity of insecticide (Clothianidin) found in dead bees after 30 minutes exposure to the dust emissions of the drilling machine.

No. - date of trial	Insecticide	Conditions after exposure	ng/bee of insecticide East	ng/bee of insecticide West	Average (ng) ± s.e.
No. 5 - 21/X/10	Clothianidin - 2010	High humidity	694 54	147 220	279 ± 142
		Lab humidity	264 527	262 1004	514.25 ± 174.7

In any case it seems that acute poisoning of bees can more probably be linked to an aerial contamination rather than to a contact with marginal vegetation. It is important to investigate the possible mechanism through which honey bees come into contact with the dust emitted by the drilling machines. Once this mechanism is clarified, it will be possible to improve drilling machines and to take measures to mitigate risk.

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CHAPTER IV

Fatal powdering of bees in flight with particulates of neonicotinoids seed coating and humidity implication.

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Fatal powdering of bees in flight with particulates of neonicotinoids seed coating and humidity implication.

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Abstract

Losses of honeybees have been reported in Italy concurrent with the sowing of corn coated with neonicotinoids using a pneumatic drilling machine. Being unconvinced that solid particles containing systemic insecticide, falling on the vegetation surrounding the sown area, could poison bees foraging on contaminated nectar and pollen, the effect of direct aerial powdering was tested on foragers in free flight near the drilling machine. Bees were conditioned to visit a dispenser of sugar solution while a drilling machine was sowing corn along the flight path. Samples of bees were captured on the dispenser, caged and held in the laboratory. Chemical analysis showed some hundred nanograms of insecticide per bee. Nevertheless caged bees, previously contaminated in flight, died only if kept in conditions of high humidity. After the sowing an increase of bee mortality in front of the hives was also observed. Spring bee losses, which corresponded with the sowing of corn coated seed, seemed to be related to the casual encountering of drilling machine during foraging flight across the ploughed fields.

Keywords

Apis mellifera, corn dressing, colony losses, clothianidin, imidacloprid

Introduction

In the last few years, honeybee colonies throughout the world have been subject to rapid losses (Underwood *et al.* 2007, vanEngelsdorp *et al.* 2009), in particular in South Europe in the order of 40% (Neumann and Carreck 2010; Mutinelli *et al.* 2010). The beehive heritage in Europe decreased from over 22.5 million in 1990 to about 15.8 million in 2009 (FAO 2011). From the 1970s to the 1980s the parasitic bee mite, *Varroa destructor*

appeared in Europe and United States, (Thompson *et al.* 2002), passed from the East Asiatic *Apis cerana* Fabricious to the African and European *Apis mellifera* L (Anderson and Truman 2000), causing losses to apiaries (Neumann *et al.* 2010; Ratnieks *et al.* 2010). Colonies infected by varroa die within 1-3 years without chemical interventions (vanEngelsdorp *et al.* 2008; Rosenkranz *et al.* 2010), which are effective in reducing the losses that are observed in autumn and the end of winter (Kraus and Page 1995; Fries and Perez-Escala 2001).

The sowing of maize (*Zea mais* L) from mid march to may, in the corn growing regions of Italy and Europe, was often accompanied by a rapid disappearance of foraging bees, sometimes with accumulations of dead bees in front of the hives. These spring time deaths of colonies are chronologically distinguishable from those caused by varroa; the latter is efficiently controlled by professional beekeepers, who on the other hand, do not know how to avoid the deaths that occur at the time of the maize sowing. During the last decade a close relationship was observed between the deaths of bees and the use of pneumatic drilling machines for the sowing of maize seeds coated with neonicotinoid insecticides (Greatti *et al.* 2003). At the end of the 1990s, soil insecticides were replaced with the coating of maize seeds (Taylor and Harman, 1990) with fipronil (Colliot *et al.* 1992; Turnblad *et al.* 1998) and the widespread neonicotinoids (Elbert *et al.* 2008) which, being systemic, penetrate the seedlings protecting them from wireworms beetles (*Agriotes* spp.), cutworms (*Agrotis* spp.) and the rootworm, *Diabrotica virgifera* LeConte (Stamm *et al.* 1985; Altmann 2003; van Rozen and Ester 2010), that are the most dangerous insect pests of maize seedlings in spring. Furthermore these insecticides also control aphids (*Rhopalosiphum spp.*, *Sitobion* spp. and *Metopolophium* spp.) and leafhoppers (*Laodelphax striatellus* Fallen) that are virus vectors. In pneumatic sowing machines the seed is sucked in, causing the erosion of fragments of the insecticide shell that are forcefully expelled with a current of air. How the insecticide comes into contact with the bees is the subject of this paper. The premiss (Greatti *et al.* 2003) was that the bees die by collecting contaminated pollen and nectar, because solid fragments of the neonicotinoid seed coating fall on the vegetation surrounding the seeded areas. This hypothesis is up to now widely accepted (Pistorius *et al.* 2009). Neonicotinoid concentrations in the vegetation at the margins of the seeded areas were shown to be about 50 ppb (Greatti *et al.* 2006; Maini *et al.* 2010), not sufficient to cause acute toxicity in foraging honeybees (Yang *et al.* 2008). Being unconvinced that bees were poisoned by the contact with the surrounding vegetation, other lethal sources in the fields were sought to justify such rapid

mortality during the spring maize sowing. A cause of acute contamination was attributed to the high concentration of neonicotinoids in the guttation drops of coated maize seedlings; these contain lethal doses of insecticide (Tapparo *et al.* 2011), sufficient to kill a bee within minutes of contact (Girolami *et al.* 2009; Riebe 2009). However, these bee deaths may not occur at the time of sowing, but after the emergence of plants when guttation drops are produced, therefore further sources of poisoning were hypothesized. In the first instance the possibility that bees could be poisoned by drinking dew and guttation drops on nearby vegetation directly contaminated by particulates during the sowing was considered but without finding acute toxicity. On the other hand, an aerial contamination of caged bees near the sowing machine in action was observed, with lethal effect if the relative humidity is high (Marzaro *et al.* 2011). In the present work attention is focused on the possibility that bees can be directly dusted in flight with fragments of shell coating emitted by a drilling machine in action, whilst flying on the usual route between the hive and food sources. High humidity was further studied as a possible key factor, increasing the lethal effects of powdering.

Material and methods

Experimental site and insect origin

Field trials took place at the experimental farm of the Agricultural Faculty (University of Padova) located in Legnaro (Padova). The plot was 50 m wide by 70 m long (coordinates: 45°20’41. 19”N-11°57’16.22”E). The Padova Beekeeping Association (A.P.A. Pad) supplied 4 hives.

Seed employed and sowing

Two batches of seed were used for the trials: one produced in 2009 and the second in 2010 hereafter called “2009 or 2010 coating” respectively. The coatings (hybrid employed X1180D 964890; Pioneer Hi-bred Italy) were: Celest XL®, containing only fungicides, (Syngenta, Basel, Switzerland; Fludioxonil 2.4% and Metalaxyl-M 0.93%),

Table 1 Free flight field trials.

No	Date	Starting time, length (min)	Active ingredient and coating year	Meteorological conditions				No. bees tested	
				t (°C)	RH (%)	Wind		Relative humidity	
						direction	Speed (m/s)	High	Laboratory
1	14/7/09	9.30-60	Clothianidin - 2009	28	65	ENE	2.5	60	60
2	23/7/09	9.00-60	Imidacloprid - 2009	28	69	E	2.4	60	60
3	15/10/09	11.00-60	Imidacloprid - 2009	13	29	ENE	3.9	60+60[a]	60
4a	02/09/10	10.30-60	Fludioxonil+Metalaxil-M-2010	21	50	NNE	2.8	60	60
4b	02/09/10	12.00-60	Clothianidin - 2010	24	46	ENE	2.7	60	60

[a] Bees collected in the front of the hive.

Poncho® (Bayer Cropscience AG., Leverkusen, Germany; clothianidin 1.25 mg/seed) (Andersch and Schwarz, 2003; Altmann, 2003) and Gaucho 350FS® (Bayer Cropscience AG., Leverkusen, Germany; imidacloprid, 0.5 mg/seed) (Elbert *et al.* 1990) (table 1). The seeds were supplied by A.I.S. (Italian seed association) courtesy of MiPAAF (Ministry of Agriculture, Food and Forestry) for the research project Apenet. The 2009 and 2010 seed batches have a quantity of dust abrasion under the limit of 3 g/ 100kg seeds. The quantity was tested with the Heubach test, considered the method which best allows stand standardization of dust abrasion measurements within the seed industry (Apenet, 2009; Nikolakis *et al.* 2009; Apenet, 2010). A Monosem NG Plus (Monosem, Largeasse-France) drilling machine was used for all the sowing operations. Normally 73,000 to 74,000 seeds per hectare were sown (75 cm between rows, 18 cm between seeds in the row). The drill moves at 6-7 km/h with a seeding width of 3 m and requires a minimum of 30 min to sow 1 ha. The air waste pipe is situated on the right hand side of the machine and expels air (and dust) at about150 l/s, at a height of 1.8 m and an angle of 45° to the horizontal. A deflector for direct air stream directed to the soil are not reported in this paper.

Conditions after exposure and influence of relative humidity

Once the bees were exposed to the insecticide powder in the field and captured (as reported in the following paragraph), they were singly transferred in small cubic (5 × 5 × 5 cm) tulle cages, in a room at a controlled temperature (22±1.5°C). Half of the cages were kept at a relative humidity lower than 70%, with the use of de-humidifier if needed, hereafter designated as lab humidity; the other half of the cages were kept at a relative humidity close to saturation (>95%), hereafter designated as high humidity. To obtain conditions of high humidity, singly caged bees were held in plastic boxes with plexiglass

on top that was sprayed with water. Moistened absorbent paper was placed under the cages which were raised above the paper to prevent the bees getting wet. The humidity was repeatedly checked with an electronic hygrometer and also with a traditional hygrometer (with dry and wet bulb). All the bees were fed with drops of honey on the top of the cage which was replaced when necessary.

Dusting trials

The progressive number, the date, the duration and the insecticides or fungicides used in the different trials are reported in table 1 along with the meteorological conditions and the number of bees tested. For the trials bees were conditioned to visit a dispenser containing a 50% (wt : vol) water solution of sucrose, placed to the north of the hives. Initially the dispenser was put close to the landing board and then moved progressively further from the apiary. The dispenser was an earth colored plate ø 0.25 m, to avoid the possible attraction of bees from other apiaries. The bees leaving from the hives, in order to reach the dispenser, had to fly over a screen house, a small vineyard for 25 m, and over a 70 m ploughed area of the plot for a total of 100 m. The sowing was carried out on the plot keeping a minimum distance of 35 m from the hives and from the dispenser. In all the trials (table 1) samples of 24 bees were collected; the first before the beginning of the sowing and 4 others at intervals of 15 min. The bees were captured at the dispenser with vials and placed singly in tulle cages, then kept, half in high humidity and half in lab humidity, for a total of 120 bees per trial. In all the trials (table 1), dead bees were counted in front of the hives 2 hours after the end of the sowing and in the evening of the same day, as well as the morning and the evening of the following days. In trial 3 (table 1), in addition to the 5 samples taken from the sugar dispenser, 5 other samples of 12 bees, employing the same timetable, were captured in front of the hives using an entomological net, successively caged and all held in high humidity (table 1).

Samples of dead bees

In trial 1, after 3 hours from the end of the sowing and the day after, 2 samples of 7 dead bees were collected from the ground in front of the apiary. Also in trial 3, samples of 8

Table 2 Number of dead bees (in groups of 12), exposed in free flight to the emissions of the drilling machine, captured at intervals of 15 min from the beginning of the sowing, caged and placed in varying RH conditions.

No.-date of trial	Insecticide	Condition after exposure	Time from start of sowing				
			0 min	15 min	30 min	45 min	60 min
1–14/07/09	Clothianidin - 2009	lab humidity	0	0***	0***	0***	0***
		high humidity	0	12	12	12	12
2–23/07/09	Imidacloprid - 2009	lab humidity	0	2***	0***	1***	3***
		high humidity	0	12	11	12	12
3–15/10/09	Imidacloprid - 2009	lab humidity	0	0***	0***	1***	4***
		high humidity	0	10	12	12	12
		high humidity	0[a]	0[a]	12[a]	10[a]	10[a]
4a–02/09/10	Fludioxonil + Metalaxil-M (Celest XL) - 2010	lab humidity	0	0ns	0ns	1ns	0 ns
		high humidity	0	0	1	0	1
4b–02/09/10	Clothianidin - 2010	lab humidity	0	1**	1***	3***	5*
		high humidity	0	7	12	11	12

[a] Bees collected in the front of the hive.
The asterisks indicate significant differences in the same trial, in respect to the successive number within the same column (***:p<0.001; ** :p<0.01; * :p<0.05).

Table 3 Content of neonicotinoids in honeybee samples collected at different times from the starting of sowing, after their flight near the drilling machine.

No - date of trial	Insecticide	Collecting site	Sampling time[a]	No. of bees analysed	Quantity of insecticide in bees ng/bee
1-14/07/09	Clothianidin - 2009	Dispenser	30 min[b]	7	674 ng/bee
		Hive	3 h[c]	7	161 ng/bee
		Hive	day after[c]	7	118 ng/bee
3-15/10/09	Imidacloprid - 2009	Dispenser	30 min[d]	4	3,661 ng/bee
		Dispenser	45 min[b]	8	442 ng/bee
		Hive	3 h[c]	8	500 ng/bee
		Hive	4 h[c]	8	53 ng/bee
		Non woven net	day after	4	29 ng/bee

[a] Time from start of sowing.
[b] Bees captured at the dispenser and dead in laboratory in high humidity.
[c] Bees found dead on the ground in front of the apiary.
[d] Bees found dead on the ground near the dispenser.

dead bees were taken from the front of the hives at 3 and 4 hours from the end of the experiment (table 3). Furthermore samples of 4 bees found dead on the ground near the dispenser were collected at 30 min from the starting of the sowing in trial 3 and on the non woven net the day after (table 3). Two other samples of apparently healthy bees that were collected at the dispenser and subsequently died inside the cage in the laboratory,

were taken for analysis (7 bees collected after 30 min in trial 1 and 8 bees after 45 min in trial 3).

Neonicotinoid content in bee samples

Samples of honeybees that died during the trials were taken for analysis of the insecticide content to the Department of Chemical Sciences of the University of Padova. The samples of bees, pooled for analysis, were stored at +2°C for few days. The treatment of the samples started with a drying process; the bees were put in a thermostatic oven, at 100°C for about 2 h. The samples were then ground with a metallic pestle, methanol was added and the samples were placed in an ultrasonic bath for 25 min. Samples were finally centrifuged, the floatage was separated and filtered with Millex HV 0.45 µm syringe filter (Millipore). The analyses were performed in a UFLC instrument (Ultra Fast Liquid Cromatography, Shimadzu XR – Prominence) equipped with an UV-Vis diode array detector and a Shimadzu XR - ODS II (2.2 µm, 2 x 100 mm) analytical column and a Phenomenex Security Guard pre-column. The following instrumental procedure was optimized: eluent flow rate of 0.4 ml/min, gradient elution (0-0.5 min 70:30% water/acetonitrile; 0.5-1.5 min linear gradient to 100% acetonitrile; 1.5-3 min 100% acetonitrile), 45°C column temperature, multiwavelength acquisition of detector signal and analytes quantification at 269 nm for Clothianidin and Imidacloprid. Instrumental calibration (external) was performed by the analysis of standard solutions in the 0.1-100 mg/liter concentration range of analytes, prepared in methanol-water solutions (50% vol : vol) from pure analytical standards (Sigma-Aldrich Group, Milan, Italy; Pestanal, purity >99.7%). Analysis of spiked samples (blank bees added with 0.5–1 µg/bee of clothianidin and imidacloprid) showed recovery factors in the range 75–105%. Methanol (VWR, International, Milan, Italy) and acetonitrile (Riedel de Haen, Sigma-Aldrich Group) were of HPLC grade. Pure water was produced by Milli-Q equipment (Millipore, Billerica, MA).

Statistical analysis

For each sample of 24 bees collected at a particular time interval, we tested the null hypothesis that the frequency of mortality occurred independently of humidity using a chi-squared goodness-of-fit test.

Results

Behavioural aspects

When the sucrose solution was poured into the dispenser it was possible, after few minutes, to see the arrival of some hundreds of experienced foragers. It was easy to observe that bees usually flew at a height of about 2 m and, normally they do not change their direction in proximity of machine only if they encountered the outline of the drilling machine they passed at a distance of few meters to the sides. Observing the bees in flight in sunny conditions a minimum of 15-20 foragers per minute over the ploughed area was calculated approximately.

Free flight dusting and humidity

To assess the influence of toxic powder from the drilling machine on honeybees in free flight, the machine was placed on the flight path between the hive and the dispenser with sugar solution. The bees captured, in the trial 1 at the sugar dispenser, at the beginning of the sowing, showed no symptoms of poisoning and none died when taken to the laboratory, either in conditions of lab or high humidity. In the subsequent 4 samples all the bees died in conditions of high humidity and none in lab humidity (table 2). After 3 h from the end of this test, an accumulation of about 400 dead bees was observed in front of the four hives which, by the end of the day after, had reached the number of 1490. On the previous days, the number of dead bees in front of the hives, were less than 50 in the apiary.

In trial 2, similar results to the previous test were obtained. None of the bees collected at the beginning of the sowing died. In the succeeding samples, only in high humidity conditions did high mortality emerge, while only 6 died (out of a total of 60 bees) of those held in lab humidity. By the evening, and different from trial 1, the number of dead bees in front of the 4 hives did not significantly increase and was lower than 50 bees.

In trial 3, no substantial differences emerged in respect of the previous trials 1 and 2 when referring to the mortality of bees collected at the dispenser (table 2). In this trial, samples of flying bees were also collected in front of the hives, then caged and all held in high humidity. No mortality was observed at time 0 and also in the sample collected after 15 min; in the successive samples high or total mortality occurred. In the evening about 300 dead bees were present in front of the hives, the day after there were about 500. None of

the 6 bees fed in laboratory with the sugar solution of the dispenser, collected at the end of the sowing, died.

In trial 4a, seeds coated just with fungicides were used and only 3 bees out of 120 tested died; no indicative difference emerged among the samples collected at the beginning and during the sowing nor between the two humidity conditions (table 2).

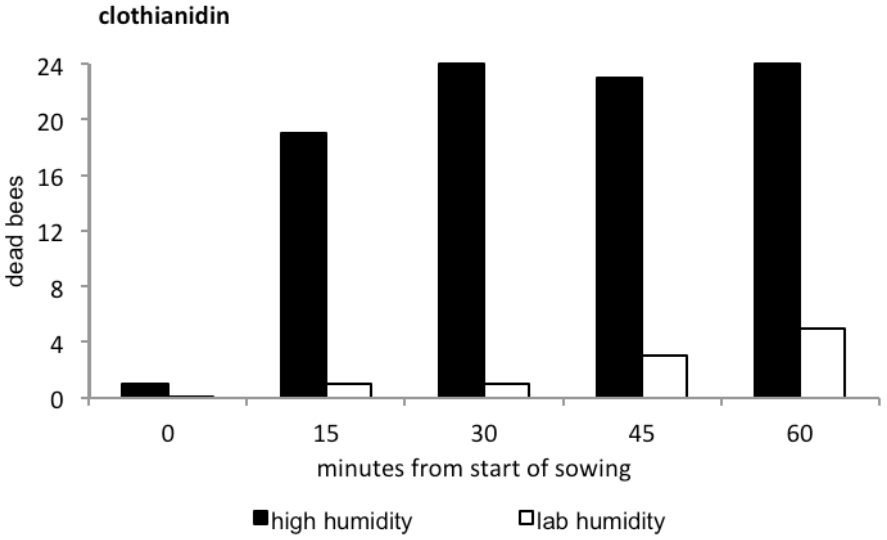
In trial 4b seeds treated with insecticide were sown immediately after the trial 4a. In the sample collected at 15 min the mortality was relatively high in high humidity and in the successive samples almost all died (table 2). A discrete mortality was also observed in samples held in lab humidity, collected after 45 and 60 min from the beginning of the sowing.

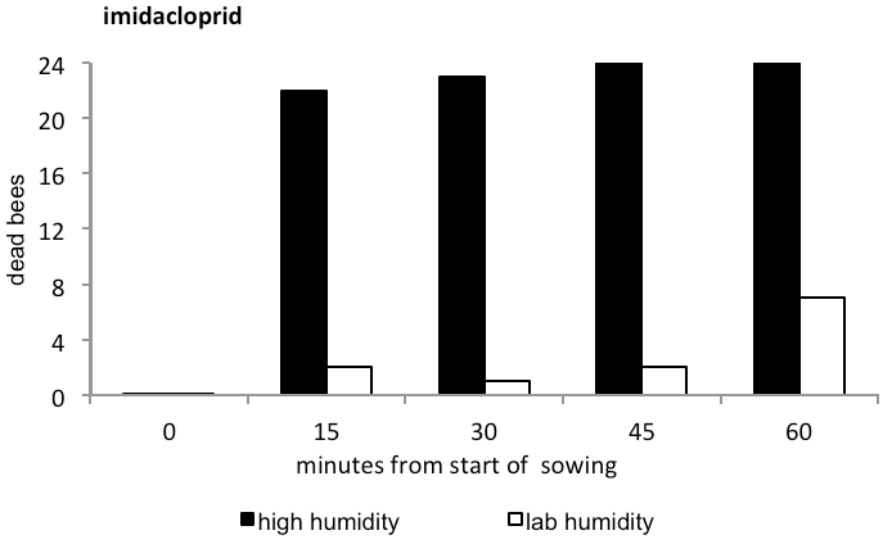
In front of the hives less than a hundred dead bees were found on the succeeding day. A highlight of the four free flight trials, when seed treated with insecticides was employed, is shown in fig.1.

Chemical analysis.

The sample of bees captured in trial 1 at the dispenser, apparently in good health, caged, transported to the laboratory and subsequently dead after some hours, showed average concentrations of clothianidin of 674 ng per bee (table 3). The sample of bees found dead 3 hours after the end of the sowing in front of the hives revealed an average of 161 ng/bee of active ingredient. In the sample of 7 dead bees collected the day after, in front of the hives, concentrations of more than 100 ng/bee (table 3) were found. In trial 3, with seed coated with a.i. imidacloprid, a sample of 4 bees found expired on the ground near the dispenser, during the sowing, contained an average of 3,661 ng/bee of imidacloprid. The sample of bees collected healthy at the dispenser during the sowing which died subsequently in laboratory showed a concentration of 442 ng/bee of a.i. The samples collected in front of the hives, in succeeding hours after the sowing, showed contents of insecticide of 500 and 53 ng/bee (table 3). The day after the sowing the dead bees collected from the non woven sheet revealed a content of about 30 ng/bee.

Fig.1 The mortality of groups of 24 bees that flew in the free flight trials near the drilling machine placed between the hives and the sucrose dispenser in four different trials with two insecticide coatings. The bees where captured, on the dispenser, every 15 min, from the starting up of the drilling machine, subsequently caged and placed in high or laboratory humidity. Each column shows the sum of bee deaths in two different experiments.





Discussion

Direct dusting in flight

The experimental results showed that the bees can be powdered with fragments of seed coat during their foraging activity when they are flying freely near the drilling machine in action. The trials reproduced the natural behavior of bees that repeatedly visit a food source, flying along the same route. Chemical analysis of the bees captured alive during the sowing at the dispenser (which died subsequently in laboratory), showed a high content of the neonicotinoid insecticide employed in the trial, with values in the order of 500 ng/bee of active ingredient. This amount is potentially lethal since the topic LD_{50} for clothianidin is 21.8 ng/bee and for imidacloprid 17.9 ng/bee (Iwasa *et al.* 2004). These amounts make sense of the almost total mortality observed in bee samples, collected at the dispenser after their flight during the sowing and held in high humidity conditions. Furthermore the use of single cages for holding bees minimizes the possibility of the contamination of bees after capture, as was observed when contaminated and uncontaminated bees are caged together (Greatti, unpublished data). The link between bee poisoning and the toxic emissions of the drilling machine is confirmed by the absence of mortality in foragers captured before the starting up of the machine (time 0; fig. 1)
and also in all the samples when only fungicide coating was used (trial 4a; table 2). This allow us to disregard other hypothetical sources of acute toxicity, for example warm air and exhaust emissions from the tractor, air born particles of soil containing residues of pesticides that could also contaminate the sugar solution of the dispenser. In fact, when this solution was offered to bees, in trial 3, no acute toxicity was observed. Furthermore, the absence of toxicity at time 0, in all the trials reported, suggest that long distance wind borne powders seed abrasions particles emanating from seed bags need further experimental approach in order to confirm their implication lethal bee poisoning.

Since bees are poisoned with neonicotinoid in flight it no longer seems important to consider, as cause of massive bees death, the moot point of contact with contaminated vegetation (contaminated pollen, dew or leaf surfaces) hypothesized in relation to the dust emitted by the drilling machine. Note that, on the basis of newly acquisitions, the presence of insecticides on pollen in the hive, is not necessarily a consequence of plant contamination before collection, but maybe due to the brushing of pollen from the bee body previously contaminated by insecticide during the flight.

The aerial powdering of bees with lethal consequences has been observed also where caged bees were forcibly exposed to the emission for half an hour (Marzaro *et al.* 2011). The research based on bees in free flight first adopted in this experimentation allows us to establish experimentally the correlation between emissions of particles from the drilling machines and bee poisoning during their foraging activity. Is noteworthy that bees collected during trial 3 in front of the hives, with an entomological net, presented a mortality similar to that of bees collected at the dispenser, this suggested that all the foragers in front of the hives had visited the dispenser; it is quite acceptable since, in October when the trial was carried out, flowers or sugar sources were not available, furthermore some contamination between bees cannot be excluded.

Data reported on poisoning in flight were obtained with an old unmodified drilling machine, in order to understand the implications of neonicotinoids (before they were banned) in bee poisoning incidents during sowing of maize in Italy (Mutinelli *et al.* 2010), the Upper Rhine Valley and in parts of South Bavaria (Pistorius *et al.* 2009). The consequences on bee survival of the attachment to sowing machines of low-drift sowing equipment (Alix et. al 2009) are not considered in this paper. Nevertheless is now possible to experimentally induce poisoning of bees in the fields and therefore consider the beneficial effects not only on drift reduction but also on bees survival. Preliminary results of the effects of the exhaust air directed onto the soil (Foster 2009), seem unclear even if improved coating (2010) is adopted (table 2).

Influence of humidity

In previous research, the influence of high relative humidity in bee deaths, after the dusting in the field with toxic emissions inside cages, was hypothesized (Marzaro *et al.* 2011). In this work, the influence of different relative humidity was further tested on bees powdered in free flight and subsequently caged. The results (table 2), confirmed that a high humidity condition is a determinant in the lethal poisoning of bees in the laboratory. The results are clear (fig.1), both with bees previously powdered with clothianidin and imidacloprid. Furthermore, no differences in toxicity emerged between the two seed batches containing clothianidin (2009 and 2010). Research is in progress also for thiamethoxam and fipronil, the other corn coating insecticides that are banned in Italy (Mutinelli *et al.* 2009). The bee samples collected without insecticide contamination, before the starting up of the machine or when only fungicides were used, do not show

mortality differences when held in different humidity in the laboratory. Therefore the high humidity condition adopted cannot be considered *per se* the cause of bee death.

Coated corn sowing has caused losses in front of the hives in the open field in trial 3 while they were less remarkable in the others trials. The lethal influence of high humidity in laboratory is clear, while in the field, the relationship between high relative humidity and bee death in front of the hives is not always evident. For example, with similar values of temperature, relative humidity and wind (direction and speed) some thousands of dead bees were registered in front of the hive in trial 1 and no increase in bee death was observed in trial 2. Probably, in addition to the simple air humidity, other environmental parameters could be considered, for example cloud movements can induce a rapid decrease of solar radiation with sudden thermal shock that can modify relative humidity both in the fields and inside the hive. The bees collected at the dispenser in free flight (trial 1) and dead in high humidity conditions were shown to be contaminated with an average quantity of 674 ng/bee of clothianidin (table 3). Being randomly divided, after capture on the dispenser, the same quantity was obviously present in insects held in laboratory humidity that all survived. This suggests that in dry condition, bees can tolerate a very high quantity of insecticide powder and can survive. Irrespective of all the parameters that can condition the lethal consequences to bees powdered with neonicotinoids, the possibility of dusting in the field of bees flying in proximity of the drilling machines with potentially lethal doses is demonstrated as highly probable.

Powdering and cleaning

The intense dusting of foragers in flight in the field, confirmed by chemical analysis, may be related to the characteristics of the integument of bees, which is adapted to harvest and retain pollens. The bees in flight could be particularly efficient in intercepting particulates, but their legs are equipped with small brushes which can be used to clean themselves. Probably bees possess a hygienic instinct, also reported for varroa (Spivak and Reuter, 1998), to rid the integument of undesired powder or fragments, maybe in flight, thus preventing the rapid contact of water soluble insecticide with the body, which allows foragers to survive in dry conditions. The sudden death of bees in spring, during maize sowing, may be related to the possibility that, in particular weather conditions, bees may get damp before they were able to rid the integument of the fragments.

Poisoning scenario

Corn is the most commonly grown crop in northern Italy. For example, in the province of Padova, out of a total of 114,000 ha of arable land, more than 50,000 ha are cultivated with maize (Regione Veneto 2008; Istat 2009). Corn fields in the North of Italy are interspersed with other herbaceous crops, orchards and gardens, as is easy to check using, for example, grid references (Google Earth®) reported for the experimental plot.

The scenario of the deaths of bees at the time of the maize sowing could be linked to the normal repeated flights of foraging bees to meadow flowers such as dandelion (*Taraxacum officinalis* L.), herbaceous crops such as winter rape (*Brassica napus* L.), flowering trees in gardens, hedges and orchards (*Prunus* spp., *Malus* spp., *Crataegus* spp., etc.). In such flights there is a probability that they will cross plots assigned for maize sowing. Taking in account that a drilling machine requires 45 min for seeding one ha, the probability of encountering the toxic cloud surrounding the drilling machine is high and will be the topic of another work.

When bees fly near the drilling machine at a height of about 2 m they get powdered with a high quantity of insecticide with lethal consequences when the humidity is high. In the North of Italy in spring these weather conditions are frequently present in the first few hours of morning sun.

It should be noted that if extended mono-cultures of maize are present, with consequent lack of flowers in spring, bees would not normally cross these large areas and thus avoid contamination. This has probably happened in France where thiametoxam maize coating is not banned, and neonicotinoid insecticides are not considered a serious problem for bees (Affsa, 2009). In Germany mortality was observed where “many small sized corn fields are located in a diverse agricultural landscape with canola fields, orchards and other bee-attractive crops” (Nikolakis *et al.* 2009).

The reason why the powder emitted by the drilling machine, independently of the synergistic effects of humidity, had such a dramatic effect on bees may have a rather simple explanation. The neonicotinoids used by farmers are diluted in water in the order of 100 ppm of active ingredients for example Dantop® (clothianidin 50%) is used to control sucking insects (Uneme, 2010), at 15 g/hl corresponding to 75 ppm. The fragments expelled during the sowing, contain more than 20% of active ingredient, that is a content of insecticide at least 2,600 times more concentrated than that diluted in water for agricultural sprays.

Finally, it is probable that in the immediate future the drilling machines will be improved in order to avoid, or drastically reduce, toxic emissions. Bees in the field seem to tolerate a relatively high powdering with neonicotinoids, this means that is not necessary to completely stop the powdering, instead it would be opportune to reduce the contamination below a probable level that incurs bee deaths. In any case, the trials reported on the bees powdered in the field with relation to the use of pneumatic drilling machines with corn seed treated with neonicotinoids, give comparable results if the bees are successively held in laboratory and this is the first clear demonstration of acute lethal poisoning, in free flight, in the field.

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CHAPTER V

Aerial powdering of bees inside mobile cages and the extent of neonicotinoid cloud surrounding corn drillers

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Aerial powdering of bees inside mobile cages and the extent of neonicotinoid cloud surrounding corn drillers

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Abstract

Sudden losses of bees have been observed in spring during maize sowing. The death of bees has been correlated with the use of neonicotinoid coated seed and the toxic particulates emitted by pneumatic drilling machines. The contamination of foragers in flight over the ploughed fields has been hypothesized. The airborne contamination has been proven, both with bees inside fixed cages around the field, and in free flight near the driller. A new trial involving mobile cages has been established and consists of making rapid passes with single bees inside cages fixed to an aluminum bar. The bar was moved by two operators at different distances from the working drilling machine. A single pass was shown as sufficient to kill all the bees exposed to exhaust air on the emission side of the drill, when bees were subsequently held in high relative humidity. The extent of toxic cloud around driller was evaluated at the height of 0.5, 1.8 and 3.5 m and proved to be about 20 m in diameter, with an ellipsoidal shape. The shape may be influenced by working speed of the drill and environmental parameters and is easily show by adding talc powder to the seed in the machine hopper.

A new driller equipment was evaluated consisting of two tubes inclined towards the soil that direct the exhaust air towards the ground. The survival rate of the bees was not substantially increased using the modified drill and was lower than 50%. Chemical analyses show up to 4000 ng of insecticide in single bees with an average content around 300 ng. Similar quantities were observed at increased distances from the modified or unmodified drillers. This new evaluation of bee mortality in the field is an innovative biological test to verify the hypothetical efficiency (or not) of driller modifications.

Introduction

In recent decades, in Europe and North America, the colonies of honey bees (*Apis mellifera* L.) were subject to catastrophic losses (Le Conte *et al.* 2010; Neumann and Carreck 2010) characterized by a common set of specific symptoms such as the rapid loss of worker bees without a related quantity of dead worker bees being found, both within, and surrounding the hives, but with excess brood in relation to adult bee populations. This syndrome was called Colony Collapse Disorder, or CCD (vanEngelsdorp *et al.* 2009) and it is linked to interactions between different causes such as parasites, in particular *Varroa destructor* Anderson *et* Trueman (that also induce viral infections), and environmental factors including agricultural insecticides (Oldroyd, 2007). The death of bees in the maize growing area of northern Italy have been linked in the first place to attacks by *Varroa destructor* particularly where the autumn and winter deaths were concerned. However, serious losses have been observed at the same time as the spring sowing of maize seed in a distinctly different time frame to those caused by varroa; losses which are not strictly attributable to CCD in as much as large accumulations of dead bees were often found in front of the hives. The cause of the rapid death of thousands of bees during the sowing of the maize seed coated with neonictinoids has been associated with foragers coming into contact with particles emitted from pneumatic drilling machines. The contamination was thought to have come from fragments falling on the vegetation at the edges of the fields (Greatti *et al.* 2003; Greatti *et al.* 2006) but chemical analysis showed the presence of rather low (p.p.b.) concentrations of insecticides (Greatti *et al.* 2003). This hypothesis was formulated following the heightened deaths which were observed in the spring of 2000 in north east Italy and, though contested as a possible cause of the deaths (Schnier *et al.* 2003) has been widely accepted up to now (Pistorius *et al.* 2009). In the context of the general uncertainty of the effect of neonicotinoids on bees, even today (Cresswell, 2011), we were unconvinced of the contamination caused by falling fragment and a new hypothesis was formulated that unknown sources of lethal poisoning could be connected to the sowing of the maize. One of the first theories formulated was that toxic guttation drops produced by the seedlings of maize could be responsible (Girolami *et al.* 2009); however the infrequent visits of foragers to such exudations did not lead us to consider guttation as the cause of such frequent and extensive deaths. It was thus thought that bees could come into contact with the particulates, not after they had fallen on the vegetation, but directly, in flight. The experimental method by which this powdering came to light

was initially to expose bees contained in fixed, single and small cages connected to poles to the dust emitted by the maize seed drill (Marzaro *et al.* 2011). Subsequently, bees were conditioned to fly over fields destined for maize by using a dispenser of sugary solution to attract them (Girolami *et al.* 2011). Both of these methods had their limitations, in as much as, the first obliged us to keep the bees exposed to the drill emissions for long periods. The second quite faithfully reproduced field conditions in which foraging bees made repeated flights over fields, where maize was being sown to visit spring flowering (dandelion, rape and orchards); nevertheless it did not furnish answers to the questions as to distance from the drill that a bee has to pass sufficient for it receive a lethal dose of dust, or how many flights were necessary before death ensued. Thus, the theory that bees could come into contact with the powder emitted from the drill when contained in cages was tested by moving them at speed. Moreover, it was verified that the results obtained were in accordance with those in previous experiments, particularly where relative humidity in the poisoning of bees is concerned. Finally, a new method was applied to test the hypothetical usefulness of modifications to the drilling machine. Currently, the efficiency of the modifications to this machine, whereby the dust emitted is reduced and directed to the ground, have been evaluated (Nikolakis *et al.* 2009; Pistorius *et al.* 2009; Biocca *et al.* 2011; Donnarumma *et al.* 2011). What is missing , however, is experimental verification of bee mortality in relation to the modified emission of dust.

Material and methods

Experimental sites.

Field trials took place at the experimental farm of the Agricultural Faculty (University of Padova) located in Legnaro. The plot was 50 m wide by 70 m long (coordinates: 45°20’41. 19”N-11°57’16.22”E)**.** The metereological data reported were collected and processed by ARPAV. The data come from the units located in Legnaro and placed at about 200 m from the plot. The wind speed measurement reported were recorded at a height of 10 m.

Table 2 - Details of field trials carried out to evaluate the toxicity on caged bees.

Number of trial – date	Starting time – sowing method[1]- driller equipment[2]		Active ingredient[3] and coating year[4]		Meteorological conditions				No. bees tested	Humidity conditions[5] after exposure
					t (°C)	RH (%)	Wind Direction	Wind Speed (m/s)		
1 – 16/7/09	11.00-s[1]	Un[2]	C[3]	09[4]	27	75	ESE	3.4	60	L-H[5]
2 – 5/8/09	15.00-s	Un	C	09	30	63	SSE	2.8	160	H
3 – 24/8/09	15.00-s	Un	C	09	28	65	SSE	2.3	160	H
4 – 26/8/09	15.00-s	Un	C	09	27	72	SSE	2.2	190	H
5 – 03/5/11	10.00-m	Un-M	F + M	10	20	57	ENE	1.1	80	H
6 – 04/5/11	10.00-m	Un-M	I	10	17	51	ENE	5,9	80	H
7 – 11/5/11	10.00-m	Un-M	I	10	24	38	ONO	2,3	80	–
8 – 20/5/11	10.00-m	Un-M	C	10	24	49	S	1.4	80	H
9 – 19/6/11	10.30-m	M	I	10	21	68	ENE	4.9	40	H
10 – 29/6/11	10.00-m	Un-M	T	10	28	47	S	2.8	80	H

[1]: s= static mode; m= mobile mode
[2]: Un= unmodified drilling machine; M= modified drilling machine
[3]: C=clothianidin; I= imidacloprid; T= thiamethoxam; F+M= fludioxonil + metalaxyl-M
[4]: 09= 2009 seed batch; 10= 2010 seed batch
[5]: L= laboratory humidity condition; H= high humidity condition

Insect origin and holding. The Padova Beekeeping Association (A.P.A. Pad) supplied 12 hives. For the trials (with caged bees) the insects were caught with a net in front of a single colony. The bees were kept in tulle mesh cages 20 cm x 20 cm x 20 cm, fed at honey drops on the top of cage and, where possible, freed in the evening and replaced each day. Later, at the time of the tests, caged bees were captured (from the 20 cm cage) in a test tube and placed in smaller cubic cages of 5 cm in tulle and again fed with drops of honey placed on the top, as reported in Marzaro et. al. 2011.

Seed employed.

Two batches of seed were used for the trials: one produced in 2009 and the second in 2010 called "2009 or 2010 coating" respectively. The coatings (hybrid employed in 2009 was X1180D 964890 and PR32G44 in 2010 both from Pioneer Hi-bred Italy) were: Celest XL®, containing only fungicides, (Syngenta, Basel, Switzerland; Fludioxonil 2.4% and Metalaxyl-M 0.93%), Poncho® (Bayer Cropscience AG., Leverkusen, Germany; clothianidin 1.25 mg/seed) (Andersch and Schwarz, 2003; Altmann, 2003), Gaucho 350FS® (Bayer Cropscience AG., Leverkusen, Germany; imidacloprid, 0.5 mg/seed) (Elbert *et al.* 1990) and Cruiser® 350FS (Syngenta International AG, Basel, Switzerland; thiamethoxam 1 mg/seed) (Robinson, 2001; Maienfisch *et al.* 2001). The seed was supplied by A.I.S. (Italian Seed Association) courtesy of MiPAAF (Ministry of Agriculture, Food and Forestry) a departure from the suspension of the use of neonicotinoids for maize seed coating in Italy for the research project Apenet. The 2009

and 2010 seed batches have a quantity of dust abrasion under the limit of 3g per 100 kg seeds. The quantity was tested with the Heubach test, considered the method which best allows standardization of dust abrasion measurements within the seed industry (Apenet, 2009; Nikolakis *et al.* 2009; Apenet, 2010; Apenet, 2011).

Drilling machines and sowing.

A Monosem NG Plus (Monosem, Largeasse-France) drilling machine was used for all the sowing operations. Normally 73,000 to 74,000 seeds per hectare were sown (75 cm between rows, 18 cm between seeds in the row). The drill moves at 4-6 km/h with a seeding width of 3 m and requires 30 min to sow 1 ha. The air exhaust pipe is situated on the right hand side of the machine and expels air (and dust) at ≈ 65 l/s (under real sowing conditions), at a height of 1.8 m and an angle of 45° to the horizontal. A modified vacuum pneumatic drilling machine was also used where the air-stream, generated by the fan (as above described) to maintain the suction pressure, which in the unmodified driller was ejected from one single outlet, was divided into two tubes (dual pipe) of 10 cm diameter and the air released close to the surface of the ground (about 20 cm). Sowing was carried out in two modes: mobile or static. The mobile mode is the standard field method while the static mode envisages the use of two tractors. The first is usually used for raise the drill above the ground and provides the power to move the air fan, the second tractor move the drilling machine at the required speed which in turn distributes the seed; in this mode the drilling machine, while still static, functions in a similar way to the usual methods, emerging seeds are collected in 4 bowls under the machine.

Direct dusting in mobile cages and influence of relative humidity

The influence of a brief dusting to simulate that of bees flying near a drilling machine in action was evaluated by means of an aluminum bar 4 m long, to which cages, each containing a single bee, were attached every 0.4 m (10 in total). The cages were numbered taking account of the progressive distances from the drill. The bar was supported at each end by a vertical pole of 2.5 m. The bar was passed by two people at a fast walking pace (6-8 km/h) by the side of the drilling machine.

Once the bees had been exposed to the insecticide dust in the field, they were transferred (inside the same cage) to a room at a controlled temperature (22±1.5°C). In trial 1 (table 1) with the drill in static mode, passes were made on the right side where the dust was expelled, with the proximal side of the bar at a minimum distance of 2 m, 4 m, 6 m, and

at a 1.8 m height from the ground. The bar was held perpendicular to the longitudinal axis of the tractor. Two passes were made with a total of 20 bees for each of the three distances. To evaluate the influence of relative humidity in this trial, half of the cages were kept at the relative humidity of the laboratory lower than 70% (with the use of de-humidifier if needed). The other half of the cages were kept at a relative humidity close to saturation (>95%), hereafter designated as high humidity. To obtain conditions of high humidity, caged bees were held in plastic boxes with plexiglass on the top and a moistened paper on the bottom (according to Marzaro *et al.* 2011). All the bees were fed with drops of honey, periodically renewed, on the top of the cage. The even numbered cages were placed in conditions of high humidity and the odd numbers in laboratory humidity. The mortality was noted every 3 hours and the data reported refers to a 24 hour period. A bee was considered dead both the arching of the abdomen and wing block were present (Girolami *et al.* 2009).

Extent of the toxic cloud

In order to make the cloud emitted by the drilling machine visible, 200 g of talc powder were added to one of the seed containing hoppers during the sowing. The extent of toxic cloud, containing solid fragments of the seed shell surrounding the drilling machine, whilst in static mode and set in a south-north direction, was evaluated using the movable bar from trials 2, 3 and 4 (table 1). The moveable bar was passed perpendicular to the longitudinal axis of the tractor, on the left and right side of the machine, and parallel to the same axis at the front and back of the machine (all four sides of the drill). These passes were made at 4 m intervals up to 16 m (4 passes each with 10 cages, each with a single bee) at three different heights: 1.8 m in trial no. 2, 0.5 m in trial 3 and 3.5 m in trial 4. At each height 4 samples of 40 bees were tested, behind, in front of, and to both sides of the tractor, giving a total of 480 bees for all the three trials. A further 10 bees were exposed to the emissions over the tractor. After the trial, the caged bees were taken to the laboratory and all placed in high humidity. To evaluate the duration of the toxic cloud (in trial. 4), 4 and 8 min after the drill had been turned off, the bar, with 10 cages attached, was moved at 1.8 m high, along the right hand side of the drill at a distance of between 2 and 6 m.

Driller modifications and bee poisoning

In trials 5, 6, 8, 9 and 10 with the drill in mobile mode, the bees were exposed (for about 30 s) to the emission of the driller (unmodified, or modified with dual pipe deflector) with the aluminum bar perpendicular to the longitudinal axis of the tractor. The people with the bar followed and passed the tractor on the right hand side (in the first 30 m of the plot). The tractor then reduced speed and waited while the people with the bar made a U-turn and again passed the machine, once more at working speed, on the left hand side. In this way the bees were twice exposed to the cloud in a similar way to foragers in free flight making a round trip over sowing area. A first pass was made between 1 m and 5m from the side of the tractor and a second pass, with another 10 bees, was made between 5 and 9 m from the tractor. The cages were numbered taking account of the progressive distances from the drill. After exposure all the bees were fed with drops of honey on the tops of the cages, which was periodically renewed; and all were placed in conditions of high humidity in the laboratory. Three neonicotinoids and a single fungicide used to coat maize seed were tested.

Content of insecticide in bees

For chemical analysis (in trial 7), after exposure the caged bees were immediately placed in a refrigerator at 2-4 °C for 15 min until complete immobility ensued. Later they were placed in a vial in a freezer at -80°C. In order to evaluate separately the powder intake on the left and right hand side of the unmodified and modified drill, the bees were exposed in a similar way to the trials above described, but after the U-turn, passes were made either on the right hand side or on the left hand side. The bees collected in trial 7 (table 1), were individually analyzed to determine the content of neonicotinoids by the method described in Girolami *et al.* 2011 and Tapparo *et al.* 2011. Of the 20 bees analyzed, the distances from the drilling machine and the side of the exposure have been taken into account.

Statistical analysis

To compare the mortality in different samples of bees, we tested the null hypothesis that the frequency of mortality occurred independently of considered parameters using a chi-squared goodness-of-fit test.

Results

Direct dusting in mobile cages and influence of high humidity

The new mode of exposure in mobile cages gave interesting results. The bees exposed in single cages with rapid passes near the drilling machines were lethally poisoned (by clothianidin) if they were subsequently held in the laboratory in high humidity (table 2). Some mortality was also observed, in bees exposed to the most intense dusting at 2 m from the drilling machine, even though kept in lab humidity (table 2). Lethal effects were observed both in round trip test (with 2 passes) and in a single pass in the trial carried out to evaluate the extent of the toxic cloud (reported in fig. 1).

Table 2. Numbers of dead and surviving bees (in groups of 10), exposed individually in mobile cages to the emissions of the drilling machine, moved with rapid passes at the right side of the drill, at progressive distances, taken to the laboratory and placed in varying conditions of RH.

No. – date of trial	Insecticide	Exposure distance from the drill	Mortality at different distances from drilling machine				Probability*
			Lab humidity		High humidity		
			dead	survived	dead	survived	
1 – 16/7/09	Clothianidin	2 m	3	7	10	0	0,001
		4 m	0	10	10	0	0,0001
		6 m	0	10	9	1	0,0003

(*) The probability are referred to statistical differences at χ^2 test within the same row (***$p \leq 0.001$).

Extent of the toxic cloud

The emissions of talc particles from the seed hoppers enabled us to have a rapid visual image of the cloud emitted by the drill. Trials 2, 3 and 4 attempted to quantify the extent of the dust cloud of particulates, emitted by the drilling machine (in static mode), with concentrations sufficient to kill bees in a single rapid pass in a mobile cage and afterwards held in high humidity conditions. Fig. 1 shows results that are obviously relative to the model of the drilling machine used, in which the air is expelled on the right side at a height of 1.8 m. The bees that passed on the right of the machine up to a distance of 6 m all died, and a very high mortality was reported up to 12 m, at all the heights tested. Mortality was encountered on the left hand side up to 8 m distance, and mostly up to a height of 2 m (fig. 1 top). Including the deaths of those bees flying above the machine, the toxic cloud extends up to 20 m, 10 m on either side of the drilling machine. In the direction of travel (fig. 1 below), at a height of 1.8 m, the (total) lethal zone extended beyond 12 m. The toxic cloud, surrounding the drilling machine showed a flattened, ellipsoidal body of some 2 to 3 m high and 20 m wide. The cloud is slightly

shifted to the right side where the air is released and to the rear of the tractor where the drill is placed. The predominating wind was blowing in a SSE direction with a wind speed averaging less than l0 km/h (table 1).

At the end of trial 4, the dust cloud remained for almost 4 min after the machine was switched off since all 10 bees died. No further toxicity was reported after 8 min.

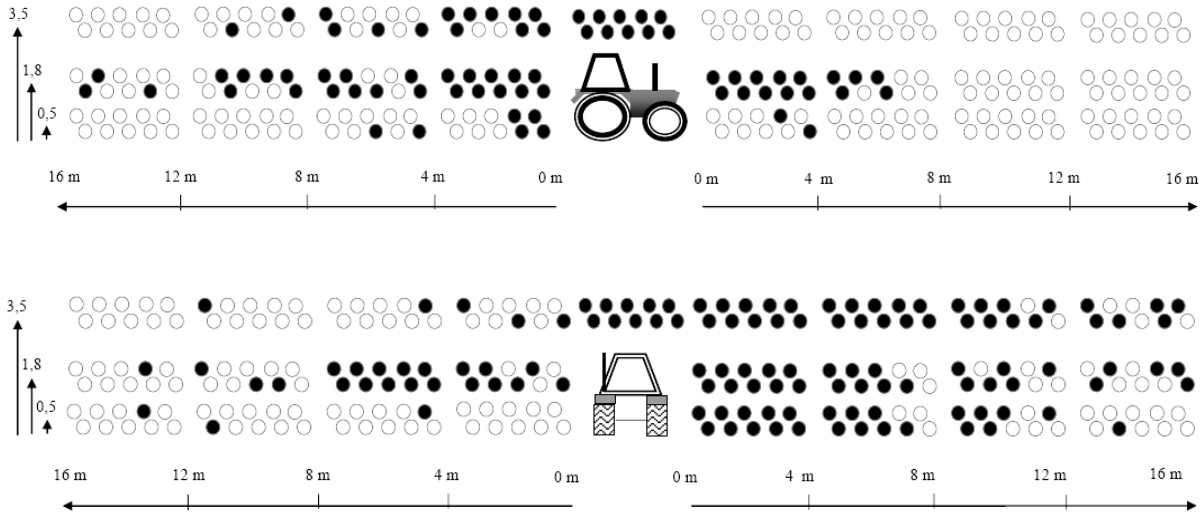

Fig. 1. The black circles represent dead bees within 24 h after a single rapid pass in mobile cages, at varying heights and distances from the drilling machine. The sowing was carried out with clothianidin coated maize seed. The top diagram shows the dead bees at the side of the machine; that below shows those in the direction of travel.

Fig. 2. Visual image, obtained by introducing talc into the hopper, of the cloud emitted by an unmodified drill (left) and a drill with dual pipe equipment (right). The modification pushes the cloud closer to the ground but is still consistent.

Driller modifications and bee poisoning

Using the mobile cage in trials no. 5-10, it was possible to evaluated the influence of the modifications made to the machine in the poisoning of bees. When seed coated solely with fungicide was employed no significant acute poisoning occurred when the bees were exposed to the emissions from the drills, for all distances and machines tested, even when they were subsequently held in high humidity conditions (trial 5, table 3). In all the remaining experiments (trials 6, 8, 9 and 10 - table 3) a relatively elevated level of

mortality occurred, above 50%, in bees passing both modified and unmodified drills at various distances. No significant differences in mortality resulted whether or not the drills were modified with the exception of trial no. 10 where, at a distance of 5-9 m, employing a drill with a dual-pipe modification a higher survival rate was observed. Amongst the different neonicotinoids tested, clothianindin appeared to be the most toxic, inasmuch as it caused total mortality in the range of 1-5 m (trial 8) and at least 80% in the other trials employed. These trials were not done at the same time and this requires that further experiments are carried out under identical environmental conditions. Using the same method the extent of the toxic cloud was evaluated.

Insecticide content in bees

In the analyzed bees (trial 7 - table 1), where the distances from the drill were taken into account, very large quantities of insecticide were found. For instance considering the bees exposed to the emission of unmodified driller, the sample that was powdered on the left hand side at a distance of 1 m showed a content of 4786 ng of clothianidin (table 4). The quantity of insecticide generally diminished in relation to the distance of exposure from the drill; not, however, in a linear manner. The minimum for the right side was 142 ng/bee at 4.5 m. As expected, the quantity of insecticide was less on the left hand side of the drill, but was still elevated and superior to the DL_{50} -18 ng of clothianidin (Iwasa *et al.* 2004). The exception was the distance of 1 m, which showed no insecticide, probably because the flow of air was hampered by the cab of the tractor.

Considering the bees exposed to the emission of modified machine on the right hand side, the quantities of active ingredient were still high, with values of half those of the unmodified drill at 1 m, similar values for samples at intermediate distances and decidedly higher approaching 9 m. On the left hand side of the drill, only those bees exposed at 6 m showed the presence of insecticide. During this trial a wind speed of 4 m/s (table 1) was blowing WSW carrying powder from the opposite side towards the tractor.

Table 3. Surviving and dead bees (groups of 20) exposed in single pass to the emission of unmodified or modified drilling machine with different insecticides at different distances.

No. - date of trial	Insecticide	Exposure distance from the drill	drilling machine equipment				probability
			unmodified (standard)		modified (dual pipe)		
			dead	survived	dead	survived	
5 – 3/5/11	Fludioxonil+Metalaxil-M	1-5 m	1	19	1	19	--
		5-9 m	2	18	0	20	--
6 – 4/5/11	Imidacloprid	1-5 m	16	4	13	7	0,298 ns
		5-9 m	11	9	16	4	0,095 ns
8 – 20/5/11	Clothianidin	1-5 m	20	0	17	3	0,052 ns
		5-9 m	17	3	16	4	0,688 ns
9 – 19/6/11	Imidacloprid	1-5 m	--	--	14	6	--
		5-9 m	--	--	8	12	--
10 –29/6/11	Thiamethoxam	1-5 m	13	7	8	12	0,118 ns
		5-9 m	13	7	4	16	0,004**

(*) The asterisks indicate statistical differences at χ^2 test between modified and unmodified driller in the same row (**p<0.01).

Table 4. Insecticide content in bees (nanograms/bee) powdered employing unmodified and modified drilling machine.

Driller equipment	Insecticide	Sampling side	Insecticide content (ng/bee) at different distances from drilling machine					mean
			1 m	2.25 m	4.5 m	6.75 m	9 m	
unmodified	imidacloprid	right side	4786	457	142	523	199	1221
		left side	<LOD*	410	110	98	33	162
modified (dual pipe deflectors)	imidacloprid	right side	2372	424	134	1778	500	1042
		left side	<LOD*	<LOD*	<LOD*	25	<LOD*	25

*LOD: lower than the limit of detection

Discussion

Direct dusting in mobile cages and influence of high humidity

The test in which fixed cages were exposed to the dusting on the margin of the sowing area (Marzaro *et al.* 2011) could have been influenced by the movement of air caused by the sowing machine, or by wind, more than and not by the flight of the bees. The adopted test method using mobile cages allowed an exposure to the dust emitted by the drill and

simulated more realistically the conditions of a bee encountering a drill in flight. Another advantage is that the exposure of the bees can be evaluated with more precision in relation to free flight, given that both the flight path and length of exposure can be controlled. The mobile cage method also assists in the evaluation of successive influences of powdering in flight in the laboratory given that the bees are already contained in cages.
The influence of high humidity in increased mortality of exposed bees has also been further confirmed with this new system of exposure, and showed no substantial differences when compared to the results obtained with the bees exposed in fixed cages (Marzaro *et al.* 2011), or in free flight (Girolami *et al.* 2011).
Riley and Ousborne (2001) reported *"…that in calm conditions, …bees typically flew with a ground speed of circa 7 m/s and we visually estimated their height of flight to be about 2 m*". Our findings agree with this reported data: the flight of bees over the ploughed area varied from 0.5 m to 4 m but was most regularly at around 2 m (unpublished data). Thus, during the sowing bees flew over the ploughed field at a height which corresponds to the toxic cloud which extends around the tractor. The exposure of the bees in mobile cage can, with reason, correspond to the exposure of a single forager in free flight when encountering a maize drill.
The speed of a bee in free flight (approximately 7 m/s, equal to about 25 km/h), is about twice that of the operators who exposed the bees in mobile cages. However this longer exposure time seem not affect in significant manner, the extent of the powdering, given that bees in free flight died in similar numbers (Girolami *et al.* 2011). In brief, the new method adopted has allowed us to establish that a single return flight in the vicinity of a sowing machine is sufficient to kill a foraging bee and on the basis of the experimentation on the extent of the toxic cloud, even a single trip.

Extent of the toxic cloud

The cloud rendered visible by the emission of talc easily documented with a camera (fig 2), may be considered a good indication of the cloud of air that contains, in suspension, the fragments of seed shell which caused the death of bees as reported in fig. 1. Take into account that talc, a silicate with a specific gravity of 2.7 is heavier than the organic material which constitutes the shell fragments (Tapparo *et al.* 2011), consequently the cloud containing the fragments of shell could be somewhat larger and last longer than that of talc. However, no substantial differences seem to exist in relation to the toxic cloud evaluated, with the mobile cage method, which clearly showed how a large lethal cloud in

the order of 20 m in diameter can form around a drill in action (fig 2), passing through which, a bee could be potentially poisoned with a fatal dose. The evaluation of the extent of the cloud was carried out with a static drill to establish the size of the cloud without the complication of the effect that forward movement would have on the emissions shape. The data obtained can form the experimental basis for further trials which take account of the speed of the drill as well as other variables such as wind speed and thermal inversion which in our observations seems to influence the thickening and the duration of the cloud at lower atmospheric levels.

The ellipsoidal dimension and compactness of the cloud assessed with the drill stationary in calm wind conditions and a hot sunny day do not necessarily correspond to the shape the cloud would take during normal sowing. Nevertheless the 20 m diameter of the cloud may be considered a realistic approximation, in that, were the drill in motion the cloud would have a narrower and more elongated shape and given a wind would be further lengthened and irregular, not centered on the drill, but logically lengthened in one direction. As a consequence the probability of a bee encountering the cloud would increase in relation to the situation shown with a static drill.

Driller modifications and content of insecticide in bees

The evaluation of the extent of the toxic cloud reported in fig. 1 was obtained by exposing caged bees to the cloud at various distances and heights. To make the contact with particles more realistic the trials were planned simulating a return flight of foraging bees.

The results obtained show that the method can also be employed to verify the effectiveness of various modifications made to drilling machines. It became evident that the advantages, by simply directing the ventilated air towards the ground, universally accepted as useful in the survival of bees (Pistorius *et al.* 2009) did not contribute in any meaningful manner to reducing deaths connected to the use of drilling machines employing coated seed. The hypothetical benefits brought about by the use of a deflector clearly contrast with the results of all the trials using a modified machine given the mortality rate still above 50% (table 3). The modifications seem, however, to bring about a small increase in survival when compared with the unmodified machine though of little relevance to the aim of defeating bee mortality. The validity of the test adopted to assess the influence of the modifications to the machine was confirmed in the chemical analysis of the caged bees passed at varying distances from the machine that were dusted with very high doses of insecticide (table 4). Clear differences of contamination with

neonicotinoids arose among the various distances and directions in relation to the drill. The chemical contamination is not conflicting with the survival results of the bees. For example, on the right hand side of the drill doses higher than 142 ng of imidacloprid could induce the death of all the bees in conditions of high humidity. This was seen in trial 1 (table 2) for bees exposed (on the right side) and then held in high humidity with total mortality. The passage of a single bee at a distance of 1 m accounted for a quantity of 4786 ng; sufficient to kill hundreds of bees, given that DL_{50} of contact with imidacloprid is 18 ng/bee (Isawa *et al.* 2004) that is 200 times less than the quantity encountered.

The modified machine has not substantially changed the values of dusting in relation to the unmodified machine. Although the quantities recovered from a single sample at 1 m were halved, the values for other distances were generally higher for bees exposed to the modified machine. In drill equipped with a "dual pipe" (and also in the other models tested - unpublished data) the exhaust air, directed towards the ground, seems displace but not reduce the toxic cloud (fig 2). The mobile cage test adopted refers to a single return flight which simulates an actual foraging flight of a bee in the vicinity of a functioning drill. It allows greater possibilities for improvement, simply for example, exposing bees for longer and in down-wind conditions to drill emissions. Moreover, the mobile cage test is a simplification in relation to the free flight test (Girolami *et al.* 2011) while still maintaining all of its validity.

At all events, the evaluation reported is a biological test based on the mortality of bees in the field and is therefore, an innovation in relation to the simple hypothetical expedient of off-crop ground deposition (Nikolakis *et al.* 2010) or of the powdering attributes of various batches of seeds using the Heubach test.

Finally, all the work reported is a further proof to explain that bees become lethally contaminated in flight. It is not necessary to take under consideration particles falling on the soil with consequent contamination of vegetation.

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CHAPTER VI

Assessment of the environmental exposure of honeybees to particulate matter containing neonicotinoid insecticides coming from corn coated seeds

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I collected and analyzed part of the data and partially drafted the manuscript.

Assessment of the environmental exposure of honeybees to particulate matter containing neonicotinoid insecticides coming from corn coated seeds

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ABSTRACT

Since seed coating with neonicotinoid insecticides was introduced in the late 1990s, European beekeepers have reported severe colony losses in the period of corn sowing (spring). As a consequence, seed-coating neonicotinoid insecticides that are used worldwide on corn crops have been blamed for honey bee decline. In view of the currently increasing crop production, and also of corn as a renewable energy source, the correct use of these insecticides within sustainable agriculture is a cause of concern. In this paper, a probable - but so far underestimated - route of environmental exposure of honey bees to and intoxication with neonicotinoid insecticides, namely the atmospheric emission of particulate matter containing the insecticide by drilling machines, has been quantitatively studied. Using optimized analytical procedures, quantitative measurements of both the emitted particulate and the consequent direct contamination of single bees approaching

the drilling machine during the foraging activity have been determined. Experimental results show that the environmental release of particles containing neonicotinoids can produce high exposure levels for bees, with lethal effects compatible with colony losses phenomena observed by beekeepers.

INTRODUCTION

In view of the evolution of farming systems associated with the increasing global food production expected to feed a growing global population, together with the greater and greater use of agricultural products as renewable energy sources,[1-5] particular attention should be given to effective strategies for the control of environmental pollutants released by crop activities. Several adverse effects have currently been associated with these emissions, such as the loss of biodiversity and ecosystem services due to an increasing use of agrochemical compounds, their effects on human health, or the contribution of greenhouse-gas emissions in agriculture to global warming (about 30%).[6] In Europe, corn crops may represent an interesting case-study for the assessment of the sustainability of future farming strategies. Corn is largely cultivated in Europe, especially in northern Italy, France, Germany and the Balkan countries, and is largely used for both human consumption and livestock feeding. Moreover, the recent government subsidies to the "green energies" are transforming corn crops into profitable energy sources. Thus, severe drawbacks could be related to the consequent increase both in atmospheric emissions from biomass transformation processes, for instance the particulate matter emissions in highly critical areas such as the Po Valley in northern Italy, and the environmental releases of substances with recognized toxic and ecotoxic effects, such as neonicotinoid insecticides that have been associated with the worldwide crisis of honeybee colonies.[7-8] In the last decade honeybee colonies throughout the world have been subject to rapid losses[7,9] in the order of 40%,[10,11] in particular in southern Europe. This phenomenon, also named Colony Collapse Disorder, represents a worldwide crisis with adverse effects both on crop production and on ecosystems. In Italy and Europe, corn sowing - from mid-March to May - was often accompanied by a rapid disappearance of foraging bees.[12,13] These spring time deaths are chronologically distinguishable from those caused by *Varroa destructor* and a close relationship was observed between the deaths of bees and the use of pneumatic drilling machines[14-17] for the sowing of corn seeds coated with neonicotinoid insecticides.[18,19] In pneumatic drilling machines, seeds are sucked in,

causing the erosion of fragments of the insecticide shell that are forcefully expelled with a current of air. The widely accepted hypothesis is that bees die by collecting contaminated pollen and nectar, because solid fragments of the neonicotinoid seed coating fall on the vegetation surrounding the seeded areas.[13,14] But neonicotinoid concentrations in the vegetation at the margins of the seeded areas were shown to be about 50 ppb or lower,[15,20,21] which are not high enough to cause acute toxicity in foraging honeybees.[22-24] More recently we have investigated other sources of contamination for bees present in the fields, which could justify such spring mortality[25-27] and very recent results seem to confirm our hypothesis that the solid particles emitted by drilling machines, and containing a high insecticide concentration, can produce a direct powdering of foraging bees in free flight accidentally crossing the sowing fields.[15-17] This acute exposure may represent lethal doses for flying bees, coherent with the colony loss phenomena observed in spring when and where corn is sown. The present paper reports on the accurate characterization of the particulate matter emitted by a drilling machine during corn sowing. A dimensional analysis of the coating particles emitted by seeds treated with different insecticides and a quantitative determination of the total concentration of insecticide present in the air at different distances from the drilling machine were carried out to assess both factor emissions during corn sowing activities and possible exposure to neonicotinoids for flying bees approaching the drilling machine. An analytical procedure was also optimized to quantify the effective contamination of single exposed bees in the field. Different geometries of the waste pipe of the drilling machine, proposed for the modification of relevant commercial models, have been tested and compared.

EXPERIMENTAL SECTION

Seeds, insecticides and bees

Seeds produced and marketed in 2008-2010 (hybrid employed X1180D 964890 and PR44G; Pioneer Hi-bred, Italy) were used for the emission tests. The seed coatings were: Poncho® (clothianidin 1.25 mg/seed, Bayer Cropscience AG., Leverkusen, Germany), Gaucho® (imidacloprid, 0.5 mg/seed; Bayer Cropscience AG.), Cruiser® (Thiamethoxam 0.6 mg/seed, Syngenta, Basel, Switzerland) and Regent® (Fipronil 0.5 mg/seed, BASF SE). All seed batches exhibited dust abrasion levels under the limit of 3 g per 100 kg seeds (tested by Heubach test[28-30]).

Four hives were supplied by the Padova Beekeeping Association (A.P.A. Pad) for the exposure tests of flying bees (*Apis mellifera, L*).

Drilling machines and the sowing area

All tests were carried out at the experimental farm of the University of Padova, located in Legnaro (Padova - Italy), in a 50 m wide by 70 m long sowing field (coordinates: 45°20'41.19"N – 11°57'16.22"E).

A Ribouleau Monosem NG Plus (4 sowing rows, Largeasse-France) drilling machine was used, as a rule, in the emission tests. The air waste pipe of the fan, which drives the pneumatic system of seed distribution, is located on the right hand side of the machine. During sowing it expels air (and dust) at ca. 230 m^3/h, at a height of 1.8 m and an angle of 45 degrees to the horizontal. In a second series of experiments, a double pipe (i.d. 12 cm., length ca. 2 m) was fitted to the original outlet to funnel the air stream to the soil. All experiments reproduced standard sowing conditions: speed 6 km/h (66660 seeds per hectare), seed distance 75 cm between rows, 20 cm between seeds in the row); considering a seeding width of 3 m, the uninterrupted sowing time was about 33 min per 1 ha.

A Gaspardo mod. Monica drilling machine (6 sowing rows, Gaspardo Seminatrici SPA - Italy), mounting a deflector at the outlet of the fan that should release the air stream directly toward the soil (without pipes), was also employed for comparison. This machine worked at 6 km/h (66660 seeds per hectare too with a distance of 75 cm between rows, and 20 cm between seeds in the row). Considering a seeding width of 4.5 m, the sowing time was about 22 min per 1 ha.

Particulate matter emission tests

Sowing tests were carried out in two ways. In standard sowing conditions, the drilling machine worked all along the field and the following samples were collected:

a) the particulate matter that falls down to the ground (dry deposition) was sampled on a series of cellulose esters filters (diameter of 185 mm, Carl Schleicher et Schull - mod. Selecta) located at the field margin, along the wind direction. The filters, contained in a plastic vessel, were humidified by water to avoid the release of sampled particles by the wind;

b) the total suspended particulate (TSP) present in the atmosphere at the field margin was sampled by US-EPA standardized procedure using Zambelli pumps (mod. ZB1 timer, Milan – Italy) operating at 20 L/min and equipped with standard 47 mm PTS filter holder and glass fiber filters (Whatmann, 47 mm);

c) PM_{10} was sampled at the field margin by a Zambelli mod. Explorer plus apparatus, operating under standardized conditions (EN 12341:1999 PM_{10} selector, flow rate 38.3 L/min, and 47 mm glass fiber filters).

Typical sampling times were 30 min for PTS and 1h for PM_{10} samples. All filters were stored at -18 °C until the laboratory instrumental analysis.

A second experimental set was realized in order to perform more accurate analytical measurements and exposure tests: in this case the drilling machine worked in a static mode (motionless in the field) but with the same sowing parameters previously detailed, using the cardan joint of a second tractor to drive the seed distribution mechanism. Emission factors were computed by measuring the concentration of the total suspended particulate matter (TSP, sampling time 5 min) emitted by the drilling machine and collected under isokinetic condition at the end of waste pipe of the fan. A standardized stainless steel isokinetic sampling line was used (EN 13284-1:2001), equipped with a Zambelli (mod. ZB1 timer) pump, 6 mm sampling inlet, 47 mm filter holder and glass fiber filters (Whatmann, 47 mm).

During the "static" sowing samples of TSP (at 5 and 10 m from the drilling machine, sampling time 30 min) and PM_{10} (at 10 m, sampling time 30 min) were collected using the same experimental condition as in standard sowing. Moreover, the size distribution of aerosol particulate matter released during the "static sowing" was measured by an optical particle counter (OPC, Grimm mod. 1.108) in the 0.23-32 µm diameter range. The instrument was placed 5 meters from the pneumatic drilling machine in order to minimize the resuspension of dust from the soil. Both the rural background and the blank values (with the drilling machine operating without seeds) were registered and then subtracted from the experimental values measured during the emission tests.

Analysis of single bees exposed to neonicotinoids

For each bee the entire analytical procedure was carried out in separate containers. Single bees found dead in the field or close to the beehive during the sowing tests were collected in a 4 mL glass vial and stored at -80 °C. Before chemical analysis the samples were maintained some hours at - 20 °C and lyophilized for 16 h in a vacuum box equipped with a high vacuum pump (Speedvac Edwards mod. ED200A). Every bee was then ground up with a metal pestle, subsequently added with 500 µL of methanol and treated in ultrasonic bath for 30 min at room temperature. The ultrasonic treatment was repeated after addition of 500 µL of water. The resulting extracts were transferred into 1.5 mL

micro-centrifuge tubes (VWR) and centrifuged for 60 min at 10000 rpm (Hettich MIKRO 120). The upper clear solutions were collected by a syringe and transferred into 1.5 mL analytical vials after filtration on 0.2 µm syringe filters (Phenomenex, RC).

An UHPLC (ultra high performance liquid chromatography) analytical method was optimized for the determination of each seed coating neonicotinoid insecticide. The method used a Shimadzu Prominence UFLC-XR chromatograph equipped with a Shimadzu SIL 20AC-XR auto sampler, Shimadzu SPD-M20A UV-Vis diode array detector and a Shimadzu XR-ODS II (2.2 mm, 2×100 mm) analytical column with a Phenomenex (ODS 4×2.0 mm) guard column. The following instrumental parameters were adopted: eluent flow rate of 0.4 mL min^{-1}, water-acetonitrile gradient elution (0-2.65 min: linear gradient from 16 to 41% of acetonitrile; 2.65-4.60 min: linear gradient to 100% of acetonitrile; 4.60-5.25 min: 100% acetonitrile), 5 µL of injector volume, 45 °C of column temperature. Detector signal at λ=215 nm for fipronil, λ=252 nm for thiamethoxam and λ=269 nm for clothianidin and imidacloprid were adopted for analytes quantification. Although in Europe thiacloprid and acetamiprid are not used for corn seed coating, they can also be separated and quantified (λ=244 nm) by the present procedure. Instrumental calibration (external) was performed by analysis of 0.05–10 mg L^{-1} standard solutions of each analyte in 50% water–methanol.

Chemicals for the preparation of the standard solutions of fipronil, thiamethoxam, clothianidin, imidacloprid, acetamiprid and thiacloprid were purchased from Fluka (Pestanal, purity >99.7% for the five neonicotinoids and >97.5% for fipronil). Methanol (VWR) and acetonitrile (Riedel de Haen) were of HPLC grade. Water was purified by a Millipore MilliQ equipment.

Analysis of the sampled particulate matter

For the determination of neonicotinoid insecticides in the particulate samples, the filters (or fraction of filter) were introduced in 10 mL test tubes, added with 2.5 mL of methanol and treated in ultrasonic bath for 30 min at room temperature. This treatment was repeated after addition of 2.5 mL of water. These solutions were directly analyzed by UHPLC, after filtration on 0.2 µm syringe filters (Phenomenex, RC), adopting the previously optimized rapid analytical procedure.[26]

RESULTS AND DISCUSSIONS

Particulates emitted by the drilling machine

Since our first experiments, conducted in 2009 with corn seeds coated with clothianidin, the fundamental observations of Greatti[14,20] have been fully confirmed: significant amounts of coating particles are effectively emitted by the drilling machine during corn sowing. Large fragments of the seed surface (ca. 1 mm, well visible around the fan outlet) were released in atmosphere through the outlet of the air flow generated in the pneumatic device of seeds distribution. Moreover, quantitative measurements carried out at the margin of the sowing field demonstrated that 1 hour of normal activity of the drilling machine can generate the dry deposition of about 280 $\mu g/m^2$ of the insecticide (with clothianidin 2008 seed coating, about half when the 2009 seeds were used) and concentrations of clothianidin in the total suspended particulate (TSP at the field margin) of 0.24 and 0.10 $\mu g/m^3$ for the two different seed coatings (2008 and 2009, respectively). In addition, analysis of PM_{10} samples collected 10 m from the field margin (ca. 60 ng/m^3 and 10 ng/m^3 of clothianidin for the 2008 and 2009 seed coatings, respectively) clearly indicated the presence of not negligible levels of micrometric particles containing the insecticide, which were emitted by the drilling machine together with the larger ones.

Although larger particles undergo rapid sedimentation (very close to the waste pipe) and in 2009-10 new types of seed-coatings (with thicker films) were introduced in Europe, as they are supposed to be more resistant to abrasion, German - before the ban on neonicotinoids - and Austrian and Slovenian beekeepers continued to report extended losses of bee colonies in spring in conjunction with corn sowing. On the contrary, no colony losses were observed in Italy, after the neonicotinoids ban. Thus, taking into account the hypothesis of a possible acute toxic effect of the emitted particles on honeybees, a series of experiments were carried out in order to better characterize these atmospheric emissions and to assess the possible exposure of honeybees to the insecticides contained in these particles in open fields.

The size distribution analysis of the emitted particles, measured by an OPC instrument during “static sowing” of corn seeds coated with clothianidin (Poncho® 2009 and 2010), revealed a typical coarse distribution ascribable to the erosion processes occurring on the seed surface. At 5 m from the working drilling machine, a significant increase in the particles concentration was registered (with respect to the blank values, Figure 1) only for particles with a diameter larger than 2 µm.

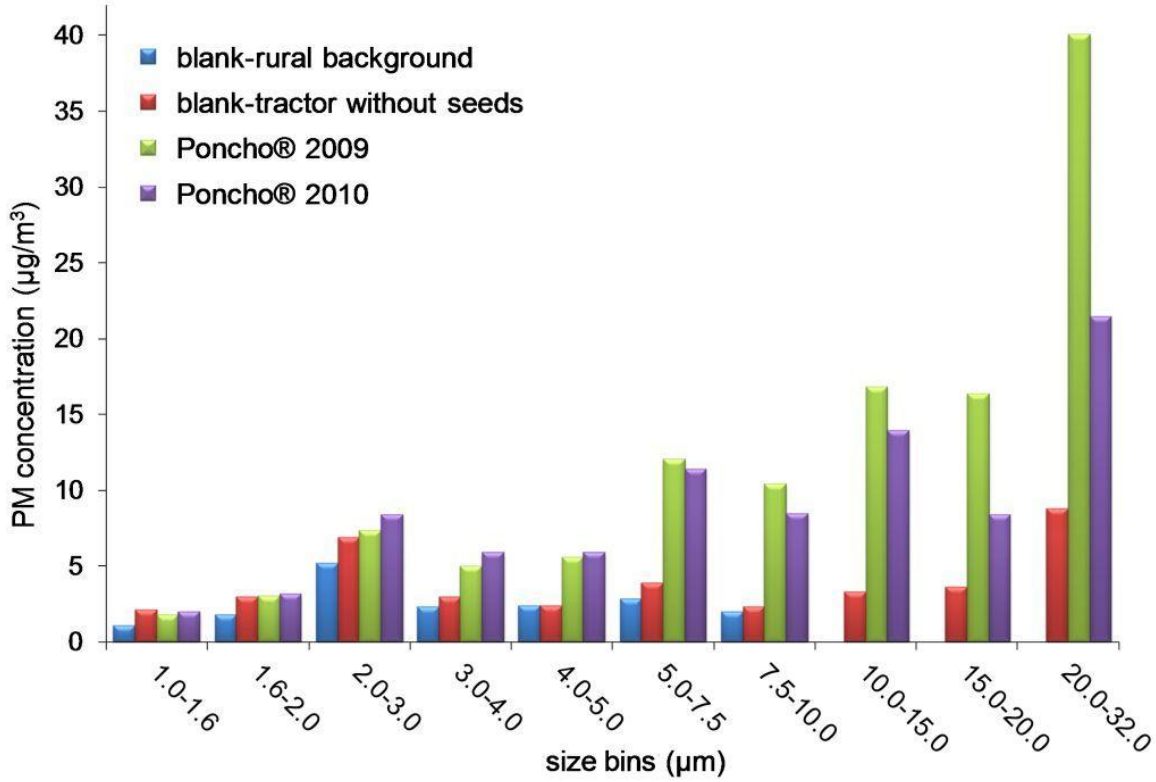

Figure 1. Dimensional distribution of particles emitted by the drilling machine during the sowing of coated seeds, measured by OPC instrumentation 5 m from the outlet of the air fan.

The mass concentration of the coating particles (estimated by the OPC at 5 m from the waste pipe, using the 2-32 µm diameter range) was 79.4 µg/m^3 for the 2009 seed coating and 49.8 µg/m^3 for the 2010 seed coating. However, in the latter case, sedimentation of very large particles (0.5-2 mm) was also observed close to the waste pipe. These results show that significant concentrations of the coating particles can surround the drilling machine during corn sowing. Moreover, they seem to indicate that the coating proposed in 2010 emits more particles, but with a larger diameter and a reduced capability to be carried by the wind (i.e. they fall to the ground near the drilling machine) compared to particles coming from the 2009 seed coating batches.

In any event, besides the larger particles emitted by the drilling machine, the presence of a significant tail of the dimensional distribution of these erosion (coarse) particles approaching the range of fine particles (few micrometers) is well evidenced for both coatings. Low vacuum SEM-EDS analysis of the sampled TSP (collected on polycarbonate filters) confirmed the presence of fine particles containing the insecticides. Of course, the environmental spreading of these fine particles is expected to be higher

than that associated with the coarse ones and, as a consequence, increased toxic effects on bees could be expected.

The effective total amount of insecticide emitted by the seed coating particles released by the drilling machine has been assessed by the analysis of TSP isokinetically sampled at the waste pipe of the fan. Our results are reported in Table 1 together with emission factors of the drilling machine estimated considering the usual sowing parameters (see Experimental). These data suggest that high quantities of insecticide are emitted during corn sowing. For instance, about 0.5 % of the clothianidin employed in Poncho 2008 and 2009 seeds (that means more than 0.4 g per hectare) is effectively released in the atmosphere as coarse particles. More recent seed coatings (2010) show higher emission factors (1.53 and 0.74 g/ha for clothianidin and thiamethoxam respectively) but, as discussed above, they are probably determined by the larger emitted particles (0.5-2 mm) that deposit quickly (very close to the air outlet) and are not carried in the atmosphere by moderate wind. Nevertheless, both OPC observation and analytical measurements in the field (see below), reveal that all kinds of seed coating release significant amounts of particles approaching the range of the fine ones and with relevant atmospheric mobility.

Analyses of the particulate matter (TSP and PM_{10}) sampled 5 and 10 m from the drilling machine (operating in static mode with different seed coatings) have also shown elevated values of the insecticide concentration in the air surrounding the working machine (Table 2). Of course, higher values are measured close to the emission source (5 m) but it is worth noticing that significant concentrations of insecticide can be observed also at a distance of 10 m from the drilling machine. Although strictly depending on wind direction and speed, these figures fully agree with the data drawn from OPC size distribution analysis: significant amounts of insecticide are emitted as few micron particles (sampled and better quantified in PM_{10}), together with the coarse ones. These particles are characterized by high atmospheric mobility and can be efficiently intercepted by the flying bees.[15-17]

Table 1. Concentration of insecticides measured at the waste pipe of the Monosem drilling machine during the sowing of corn coated seeds and relevant emission factors estimated using normal sowing parameters[a].

Corn seed, insecticide	Insecticide concentration at the outlet of the air fan (mg/m^3)	Emission factors		Fraction of released insecticide (%)
		g/h	g/ha	
Poncho 2008, Clothianidin 1.25 mg/seed	3.6[b]	0.83	0.46	0.55
Poncho 2009, Clothianidin 1.25 mg/seed	3.39 ± 0.47	0.78	0.43	0.52
Poncho 2010, Clothianidin 1.25 mg/seed	12.0 ± 1.2	2.76	1.53	1.84
Cruiser 2010, Thiamethoxam 0.6 mg/seed	5.8 ± 1.5	1.33	0.74	1.85
Regent 2010, Fipronil 0.5 mg/seed	3.57 ± 0.46	0.82	0.46	1.37

[a]Data obtained from the analysis of three independent samples (isokinetic TSP) collected during "static sowing" experiments using the Monosem drilling machine. Sowing conditions: speed 6 km/h, 4 rows of seed distribution, distance between rows 75 cm, seeds distance 20 cm (66660 seeds/ha), air flow 230 m^3/h.
[b] Value obtained from a single sample collected during the preliminary tests.

Table 2. Concentration of neonicotinoid insecticides in the particulate matter sampled near the drilling machine during the sowing of corn coated seeds[a]

Corn seed, insecticide	Drilling machine and air fan outlet modification	Distance from the outlet of the air fan		
		5 m	10 m	
		Insecticide in TSP (μg/m^3)	Insecticide in TSP (μg/m^3)	Insecticide in PM_{10} (μg/m^3)
Poncho 2009, Clothianidin 1.25 mg/seed	Monosem, unmodified	28.4	13.1	nd
Poncho 2009, Clothianidin 1.25 mg/seed	Monosem, dual pipe outlet	4.7	1.2	0.2
Poncho 2010, Clothianidin 1.25 mg/seed	Monosem, unmodified	15.0	5.3	nd
Poncho 2010, Clothianidin 1.25 mg/seed	Monosem, dual pipe outlet	6.1	1.5	1.2
Poncho 2010, Clothianidin 1.25 mg/seed	Gaspardo, mod. Monica	4.5	0.8	0.2
Cruiser 2010, Thiamethoxam 0.6 mg/seed	Monosem, unmodified	4.2	1.0	0.6
Cruiser 2010, Thiamethoxam 0.6 mg/seed	Monosem, dual pipe outlet	7.2	2.8	1.6
Regent 2010, Fipronil 0.5 mg/seed	Monosem, unmodified	12.0	1.9	0.5

[a]Average values of three independent samples and determinations. Uncertainty (standard deviation) ca. 5%. nd: not determined.

Data in Table 2 also show that, during sowing, the Poncho® 2009 corn seed coating seems to produce more particles than its 2010 version, although a higher factor emission was found for the latter. This discrepancy could be explained considering that a significant fraction of the 2010 coating is released as very large particles that cannot be easily transported to the sampling TSP apparatus (5 or 10 m). In conclusion, the two kinds of coating show a different behavior toward surface erosion and, during sowing, the 2009 version produces a more concentrated cloud of fine-coarse particles surrounding the drilling machine.

As for the modification of the air fan outlet in the attempt to reduce the environmental release of the particles containing the insecticide, we must underline that the strategies so far proposed often consist in the mere application of a pipe (or a deflector in the Gaspardo

model) that funnels the air flow toward the ground.[31] Of course, taking into account the size and the aerodynamic properties of the particles described above, it is easy to foresee the limited efficiency of this apparatus. In any case, we modified the waste pipe of the Monosem drilling machine as proposed by AFSSA[32,28] using a dual pipe that splits the air flow into two components, both downward directed and released at 20 cm from the soil. Experimental results (Table 2) confirm a reduction of the clothianidin concentration measured at the modified drilling machines (for both the modified Monosem and Gaspardo) compared to the unmodified Monosem. On the other hand, improvement has not been observed using the seeds coated with thiamethoxam. Anyway, it seems clear that the modified drilling machines also emit large amounts of micrometric particles of ecotoxicological relevance, whose acute effects on flying bees have been recently well illustrated.[15-17,33] Regarding other relevant properties of these particle clouds (i.e. their spatial and temporal dimension), although preliminary information have been acquired by toxicity data (ca. 15 m around the drilling machine; few minutes after sowing was completed)[15,17], we are aware that more detailed experiments are needed.

Analytical method for single bee analyses after field exposure

Since the first sowing tests with both static and normal operating drilling machine we observed the death of a significant number of bees whose beehives were ca. 100 m far from the sowing field. Short term mortality and the characteristic symptoms of neonicotinoid neurotoxicity[25,34,35] gave rise to the hypothesis of a direct acute exposure of the flying bees to the emitted particles as they approached the drilling machine, rather than an indirect contamination *via* the vegetation (pollen, nectar, dew) surrounding the sown area. Therefore, a series of specific exposure experiments were carried out using both caged bees positioned at various distances from the air outlet[15,17] and foraging bees conditioned to fly over the sowing field to visit a dispenser of sugar solution.[16]

In this connection, an analytical method for the determination of the insecticide content in a single bee has been optimized and validated, taking into account the advantage of the rapid UHPLC procedure recently proposed for the analysis of corn guttation drops.[26] In the present procedure, the lyophilized sample (a single bee) was grounded, extracted with methanol and analyzed by a UHPLC-DAD instrumental method that allows the complete elution of the neonicotinoid insecticides of interest, and of fipronil, in about 6 min. The method shows excellent precision: repeatability, from replicate analyses of real samples, was better than 4% for concentration levels higher than 200 ng/bee of each insecticide (4-

8% at 50 ng/bee). Although an instrumental limit of detection (LOD) of ca. 2 µg/L has been computed for each neonicotinoid insecticide from the parameters of the analytical calibration function (by the procedure suggested by IUPAC[36]), experimental uncertainties measured in the analysis of real samples indicate a reasonable LOD of ca. 10 ng/bee for the complete analytical procedure. Very limited chromatographic interferences for the UHPLC-DAD method were observed in the analysis of spring-summer sampled bees and recovery tests, using spiked samples (blank bees added with 50-200 ng/bee of thiamethoxam, clothianidin and imidacloprid), showed satisfactory recovery factors in the range 78-104%. A slightly worse chromatographic resolution (that gave higher uncertainties and lower recovery factors) was observed in the analysis of winter samples and in the quantification of fipronil.

Compared with the performance of HPLC-MS methodologies,[37,38] the LOD of the UHPLC-DAD method appears to be quite elevated. Nevertheless, the optimized procedure is rapid enough, uses a simpler instrumentation and both accuracy and LOD are adequate for the purpose, i.e. the analysis of single bees after the acute exposure to particulates containing neonicotinoid insecticides.

Insecticide content in exposed bees

Application of the analytical method to the analyses of single bees directly exposed in the field to the emitted particles has always evidenced elevated levels of the insecticide content. Although the assessment of a reliable correlation between the insecticide amounts emitted by the drilling machine and the bee uptake requires a more rigorous experimental approach than that adoptable in the field (i.e. a dedicated exposure chamber, a wind tunnel or an isolated laboratory for emission tests as that set up by Pochi and coworker[31]), the analyses of single bees sampled during the field sowing experiments revealed important information on both the effective bee exposure and the insecticide uptake mechanism.

For instance, foraging bees induced to fly over the sowing field to reach a sugar dispenser, here captured at the end of the sowing experiment (Poncho® 2010, sowing time 1 h) and maintained in laboratory under high humidity condition until death,[16,17] shown concentration of clothianidin in the range 78-1240 ng/bee (n=5, mean 570 ng/bee). A wide spread of values was also observed using Cruiser® 2010 seed coating: 128-302 ng/bee of thiamethoxam (n=4, mean 189). Taking into account the satisfactory precision of the optimized analytical procedure, this high variability is probably due mainly both to

the different number of flights over the field (or different paths approaching drilling machine) that each bee has completed before being sampled and to the effect of probable cleaning processes (dust off) occurring in flight or inside the hive. For this reason, strong dependence of the insecticide concentration on the sampling time (during sowing) has never been observed. On the other hand, in partial confirmation of the cleaning processes, non negligible differences in insecticide concentration were observed in bees captured at the dispenser and maintained, until death, under different humidity conditions[16]. Thus, after 30 min from the start of the Cruiser® 2010 sowing, thiamethoxam concentration was 267±59 ng/bee (n=5, humidity >95%) and 104±87 ng/bee (n=5, humidity <70%); using Regent® 2010 seeds, fipronil concentration was 850±330 ng/bee (n=4, humidity >95%) and 210±160 ng/bee (n=6, humidity <70%). Despite their high (but justified) variability, these concentrations well support both the bee mortality data obtained by Girolami, in which a strong dependence on the air humidity was reported,[15-17] and the hypothesis of a contact uptake in flight of the insecticide through the bee tegument, facilitated by the humidity.

The effective and lethal powdering of the flying bees has also been confirmed by quantitative measures of the insecticide "lying" on the bee surface. At the end of a sowing with Poncho® 2009 (1 h), several dead bees were found at the sugar dispenser and immediately frozen. Before the analysis, 7 bees were externally washed with methanol (15 min, in ultrasonic bath) and then analyzed by the optimized procedure. The results revealed an external concentration of clothianidin of 396 ng/bees and a total concentration of 674 ng/bee. Dead bees sampled at the hive subsequent to the end of sowing (3 h, n=7; 24 h, n=14) showed a significantly lower content of insecticide: the external concentration was always below the LOD while total levels of 155 and 119 ng/bee were measured on the bees sampled after 3 and 24 h, respectively. A similar decreasing trend was also observed after exposure of the flying bees to other neonicotinoid particulates. For instance, using Gaucho® 2009, external concentrations of imidacloprid up to 3000 ng/bee have been detected in the bees collected at the end of the sowing (240 ng/bee after 2 h, <LOD after 24 h); the total concentrations were 3650 and 325 ng/bee for bees sampled at the end of sowing and after 2 hours, respectively (<LOD after 24 h). These results appear to be very informative: they confirm (i) the elevated capability of the flying bees approaching the drilling machine to intercept the suspended coating particles, (ii) the effective lethal contamination of bees with the insecticide that

can be taken up by contact and (iii) the possible partial removal of the particles during the foraging activity or in the hive.

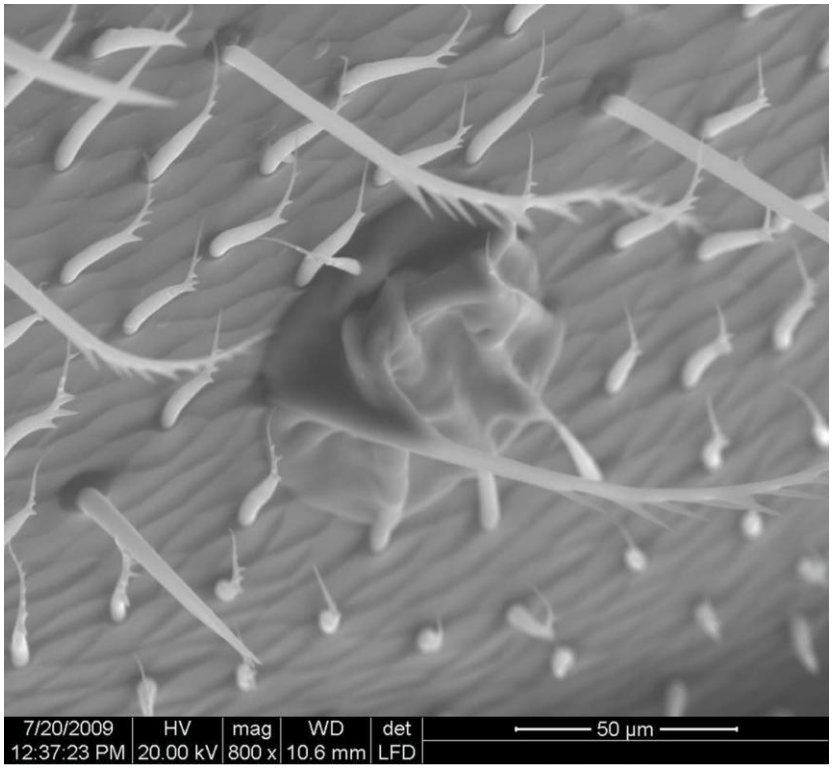

Fig. 2. Low vacuum SEM image of a seed coating particle (Poncho® 2009) that, partially modified by the air humidity, adheres to the abdomen tegument of a bee exposed to the drilling machine emissions.

The presence of coating particles on the abdomen of the flying bees, and the related uptake mechanism of the insecticide, has also been confirmed by electronic microscopy (low vacuum SEM analysis, Figure 2) observing modified coating particles that adhere to the bee tegument. This modification, that is reasonably influenced by air humidity, could explain the different toxic effect observed on powered bees maintained at different condition of humidity.[16] Moreover, it supports the experimental data that indicate a major self capability of bees to remove coating particles (finding lower concentrations) when maintained, after exposure, under normal humidity condition.

Short time exposure of single caged bees to the air flow emitted by the fan of the drilling machine (about 30 s, simulating 1-2 flight across the sowing field at different distance from the drilling machine) always induced acute lethal effects toward the bees, more evident if the exposed bees are maintained, until death, under high humidity conditions.[15-17] According to the observed toxic effects,[17] elevated levels of insecticide were always measured. For instance, caged bees exposed at different distance from the air outlet of the

Monosem drilling machine (1-9 m, using Poncho® 2010, in absence of dominant wind) evidenced concentrations of clothianidin significantly higher for bees exposed on the right side (in front of the waste pipe) with respect those exposed on the left side of the machine (Table 3). As expected for the latter ones, the dependence of the concentration on the distance from the drilling machine is not clear, as an effect of the turbulence of the air surrounding the working drilling machine. At the same time, this turbulence can also explain the variable values measured by using the modified Monosem drilling machine (with a dual pipe outlet releasing particles, downward to the soil, from both sides): actually, only a concentration range of clothianidin (71-434 ng/bee, n=10, mean 197±129 ng/bee, humidity <70%; 70-446 ng/bee, n=9, mean 216±141 ng/bee, humidity >95%) can be reasonably furnished as representative of the caged bees exposed in the 1-9 m range from the back of the machine (toward the wind direction, 1-2 m/s), without correlation with the distance.

Table 3. Clothianidin concentration in caged bees exposed, for 30 s at different distance (both right and left hand side), to the air flow emitted by the Monosem drilling machine during the sowing of Poncho® 2010 seeds.

distance from the air outlet (m)	concn detected in bees exposed on the right side (ng/bee)[a]	concn detected in bees exposed on the left side (ng/bee)[a]
1.00	1393.6 ± 0.6	115.3 ± 0.6
2.25	808 ± 2	80.7 ± 0.6
4.50	64 ± 4	110 ± 1
6.75	164 ± 4	598.7 ± 0.6
9.00	100.5 ± 0.7	25 ± 1

[a]Average values and standard deviation of the instrumental measurements (n=3) on single bee samples

According to the high insecticide levels measured in air around the drilling machine (Table 2), huge contents of insecticide have been measured in the dead bees collected at the beehive after the sowing experiment also using the modified drilling machine. For instance, the sowing (1.5 h) of Poncho® 2010 corn seeds by the Gaspardo drilling machine (with the outlet air flow directed downward by an external deflector) induced the rapid death of more than 200 foraging bees flying across the sowing area, revealing a clothianidin content in the range of 0.5-11 µg/bee. It is worth noticing that a significant decrease in the insecticide content seems to be evidenced when the sampling of the bees is delayed after death. In the hypothesis that the metabolic degradation of the insecticide

(probably effective also *post mortem*) may affect the concentration experimentally found in real samples, to such an extent that very low levels could be found also after significant exposure, specific research is in progress in our laboratory.

In conclusion, particulate matter released by the drilling machine during the sowing of corn seeds coated with neonicotinoid insecticides, represent a significant mechanism of environmental diffusion of these insecticides. Bees flying over the sowing field and approaching the emission cloud of the drilling machine can efficiently intercept the suspended particles being directly contaminated with elevated dose of insecticide, significantly higher than the LD50 values estimated for contact, with the cuticle, administration (18, 22 and 30 ng/bee for imidacloprid, clothianidin and thiamethoxam, respectively[39]). The consequent acute lethal effect evidenced in all the field sowing experiment can be well compared with the colony loss phenomena widely reported by beekeepers in spring and often associated to corn sowing. Analytical results regarding factor emissions, air concentration of insecticide around the drilling machine and consequent bee contamination, reveal that all kinds of the tested seed coatings (also those more recently proposed) do not prevent the dispersion of large amounts of fine particles containing the insecticide, producing lethal exposure of flying bees. Moreover, the modifications of the air outlet of drilling machines so far adopted seem to have a limited effect on both the factor emission and the effective bee contamination.

This emission source of particles with acute toxic effects on bees (and on other insects too) is of concern for both apiculture and crop productions based on bee pollination. But it is also a widespread ecological problem that, in view of the worldwide increase in corn production partly promoted by government subsidies to renewable energy sources, and the consequent predictable exacerbation of the problem, should require a deeper analysis of the related agricultural policies. In this connection, immediate contributions for the reduction of atmospheric factor emissions of neonicotinoid insecticides should come from studies oriented to the realization of suitable devices for an efficient reduction of toxic particles inside the seed distribution mechanism of drilling machines, and supported by quantitative data both on particulate emissions and biological effects on honeybees.

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CHAPTER VII

UHPLC-DAD method for the determination of neonicotinoid insecticides in single bees and its relevance in honeybee colony loss investigations

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Analytical and Bioanalytical Chemistry

UHPLC-DAD method for the determination of neonicotinoid insecticides in single bees and its relevance in honeybee colony loss investigations

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Abstract

In the understanding of colony loss phenomena, a worldwide crisis of honeybee colonies which has serious consequences on both apiculture and bee pollination dependent farm productions, an important role can be played by analytical chemistry. For instance, rapid and accurate analytical procedures are currently required to better assess the effects of neonicotinoid insecticides on honeybee health. Since their introduction in agriculture, neonicotinoid insecticides have been blamed for being highly toxic to honeybees, possibly at ng/bee level or lower. As a consequence, most of the analytical methods recently optimized have focused on the analysis of ultra-traces of neonicotinoids using LC-MS techniques to study the effects of sub-lethal doses. However, recent evidences on two novel routes - seedling guttations and seed coating particulate, both associated with corn crops - that may expose honeybees to huge amounts of neonicotinoids in the field, with instantly lethal effects, suggests that selected procedures need optimizing. In the present work, a simplified UHPLC-DAD method for the determination of neonicotinoids in single bees has been optimized and validated. The method ensures good selectivity, accuracy and adequate detection limits which make it suitable for the purpose, while maintaining its ability to evaluate exposure variability of individual bees. It has been successfully applied to the analysis of bees in free flight over an experimental sowing field, therefore exposed to seed coating particulate released by the pneumatic drilling machine.

Keywords: Honeybees, neonicotinoid insecticides, liquid chromatography, QuEChERS extraction, mass spectrometry.

Introduction

A recent, invasive syndrome affecting honeybee (Apis mellifera, L.) colonies in the Northern hemisphere, named colony collapse disorder (CCD), is characterized by a sudden, massive disappearance of honey bees from the hive [1-5]. Although several causes have been hypothesized, pesticides have received more consideration by the scientific community. Nowadays, experimental evidences for an association between the colony loss phenomena, including those occurring in early spring, and the use of neonicotinoid insecticides, in particular as seed dressing in corn crops, an agricultural practice used worldwide, are extensive and there is sufficient mechanistic understanding to put the question of causality beyond reasonable doubt [6-13]. Although spring mortality is characterized by a rapid disappearance of bee colonies (a typical short term effect), scientific efforts were in most cases based upon exposure to sub-lethal doses of neonicotinoids, which may weaken the colonies and make them more susceptible to both common and new diseases [10, 14-19]. In fact, since Greatti *et al.* [20] demonstrated the possible release of seed coating insecticides through the fan drain of the pneumatic drilling machine during corn sowing operation and hypothesized bee exposure to the neonicotinoid containing particles falling off to the vegetation at the field margin, experimental results showed that neonicotinoid content in nectar and pollen collected from the surrounding vegetation were always around 50 ppb or lower [12, 21-23], while higher doses are necessary for an acute toxic effect [13, 16-18, 24]. In this connection, Girolami and co-workers have recently proposed two novel routes of exposure to and intoxication with neonicotinoids which may justify such a sudden spring mortality: the translocation of a significant amount of neonicotinoids from the coated seed to the guttation drops of young corn plants [6, 24] and the direct powdering with neonicotinoid containing particles of foraging bees in free flight accidentally crossing the sowing fields [11, 21, 25, 26]. Exposure and monitoring studies also promoted several analytical methods [27-31], mainly using liquid chromatography coupled with mass spectrometry (HPLC-MS) [32-35], for the determination of neonicotinoid insecticide content in exposed bees. In these methods, but also in methodologies for the analysis of simpler matrices of interest (i.e. honey [36, 37], fruits [38-42] or vegetables [41-47]), a great effort has been devoted to both extraction and clean-up procedures that precede

instrumental analysis [48]. In order to obtain satisfactory recovery factors for both neonicotinoids and their main metabolites, several versions of the QuEChERS (quick, easy, cheap, effective, rugged and safe) method originally proposed by Anastassiades *et al.* [49] were developed [33, 35, 37, 40, 46]. Although sample pretreatment, preconcentration and HPLC-MS analysis guarantee good analytical performances - in term of accuracy, selectivity and instrumental sensitivity - lower detection limits (below 1 ng/g or 0.1 ng/bee) were always obtained applying the optimized analytical method to a large sample: typically 2-15 g, ca. 20-150 bees. In this way, information on single bee contamination was lost and only an average assessment of the low levels of insecticide uptake was made. On the other hand, as aforementioned, latest studies have demonstrated that foraging bees can be directly exposed to high (and lethal) concentrations of insecticides in the field. Corn sowing using pneumatic drilling machines and seeds coated with neonicotinoids (an agricultural practice used worldwide) release in the atmosphere large amounts of coating particles that are efficiently intercepted by foraging bees flying over the sowing fields [11, 12, 21, 25, 26]. Bees exposed to these toxic particles show characteristically acute effects, with a short term mortality which compares well with the colony loss phenomena observed by beekeepers in spring, and associated to corn sowing. Moreover, young corn plants obtained from coated seeds produce guttation drops containing high concentrations of the coating insecticide (up to 1150 mg L-1 for thiamethoxam [6, 24]), lethal for bees or other insects that may use guttations as source of water. It is worth noticing that these acute exposures to neonicotinoids, and their lethal effects on honeybees, can be easily studied and quantified by using dedicated analytical methods based on simpler instrumentation and more rapid procedures than those optimized for the studies of sub-lethal effects. For instance, neonicotinoids in guttation drops are directly analyzed by ultra-high performance liquid chromatography with diode array detection (UHPLC-DAD) [6, 24], using a rapid method which can also be easily applied (after adequate sampling procedure) to characterize particulate matters emitted by drilling machines during the sowing of corn coated seeds [11]. First attempts to use this approach in the analysis of single bees were successful, even if some chromatographic interferences emerged [11]. A UHPLC-DAD method was successfully used in the assessment of acute exposure to seed coating particulate, and consequent lethal contamination, of bees flying close to the drilling machine in the sowing field [21, 25, 26].

In the present paper, a simplified analytical method has been optimized based on QuEChERS extraction and clean-up and on UHPLC-DAD instrumental analysis for the accurate determination of neonicotinoid insecticides in single bees. Method validation was also carried out and analytical performances assessed by comparing results with those obtained by an independent UHPLC-Q-TOF-MS analytical procedure.

Experimental

Materials and instrumentation

Analytical grade magnesium sulfate (anhydrous, 99%; VWR - AnalaR NORMAPUR, Milan, Italy), sodium acetate trihydrate (99.0%, Fluka, Milan, Italy), Amberlite XAD-2 resin (Restek Ultraclean, Bellefonte, PA, USA) and primary-secondary amine sorbent (PSA, Supelco Supelclean, Milan, Italy) were used in the sample pre-treatment step. Methanol (VWR) and acetonitrile (Riedel de Haen, Seelze, Germany) were of HPLC grade and water was purified using Millipore MilliQ (Vimodrone, Milan, Italy) equipment. Pure chemicals for instrumental calibration (Pestanal, purity >99.7% for thiamethoxam, N-desmethyl thiamethoxam, clothianidin, imidacloprid, acetamiprid and thiacloprid and >97.5% for fipronil) were purchased from Fluka.

UHPLC-DAD analysis was optimized on a Shimadzu (Milan, Italy) Prominence UFLC-XR chromatograph (SIL 20AC-XR auto sampler, CTO-20A column oven, SPD-M20A UV-Vis diode array detector). LC-Q-TOF MS analysis were performed on a UHPLC system (Series 1200, Agilent Technologies, Palo Alto, CA, USA), consisting of vacuum degasser, autosampler and a binary pump coupled with both DAD and Q-TOF MS mass analyzer (Agilent Series 6520), equipped with an electrospray ionization interface (ESI), operating in dual ESI mode, with the following operation parameters: capillary voltage 4000 V, nebulizer pressure 40 psi, drying gas 10 L/min, gas temperature 350 °C, fragmentor voltage 120 V (180 V in the negative ESI mode). On both chromatographic systems a Shimadzu XR-ODS II analytical column (2.2 µm, 2.0×100 mm) and a SecurityGuardTM ULTRA cartridge, UHPLC C18 2.1 mm (Phenomenex, Castel Maggiore, Bologna, Italy) guard column were utilized.

Bees exposure tests

Bees (Apis mellifera, L.) from four hives, supplied by the Padova Beekeeping Association (A.P.A. Pad), were used in field exposure tests with particulate matter emitted by a drilling machine during corn sowing. All tests were carried out in a sowing field of the experimental farm of the University of Padova (Legnaro, Padova – Italy; coordinates: 45°20'41.19"N - 11°57'16.22"E) using a Ribouleau Monosem NG Plus drilling machines under the experimental condition already described elsewhere [11,25,26]. Commercially available corn seeds (hybrid X1180D 964890 and PR44G; Pioneer Hi-bred, Italy) produced and marketed in 2010-2011 were used; the seed coatings were Cruiser® (Thiamethoxam 0.6 mg/seed, Syngenta, Basel, Switzerland), Poncho® (clothianidin 1.25 mg/seed, Bayer Cropscience AG., Leverkusen, Germany) and Gaucho® (imidacloprid, 0.5 mg/seed; Bayer Cropscience AG.). A neonicotinoid insecticide in granular form for soil treatments was also used (Santana, containing clothianidin 0.7 %, Sumitomo Chemical Agro Europe, Saint Didier au Mont d'Or, France).

Bees flying over the sowing field, or found dead in the field or close to beehives during the sowing tests, were collected in 1.5 ml test tubes and stored at -80 °C.

Single bee extraction and clean-up

Sample pre-treatment was carried out by a simplified QuEChERS procedure. In a 1.5 ml test tube each bee was treated with 100 µl of water, 500 µl of acetonitrile, roughly pounded with a metal pestle and then added with 30 mg of magnesium sulphate and 5 mg of sodium acetate. The sample was then placed in ultrasonic bath (ELMA® Transsonic Digitals) for 15 min at room temperature and then centrifuged for 15 min at 10 000 rpm (Hettich MIKRO 120). The supernatant was collected by a syringe, transferred into another test tube and added with 20 mg of PSA sorbent or Amberlite XAD-2 resin. In order to get a quantitative recovery of the analytes the extraction/clean-up process was repeated treating the bee residue with other 500 µl of acetonitrile; after centrifugation, extracts were unified, evaporated to dryness at 40 °C under a nitrogen flow and the residue was dissolved with 300 µl of a water/methanol solution (90:10). The final extract was then centrifuged for 15 min at 10000 rpm, filtered on 0.2 µm syringe filter (Phenomenex, RC) and transferred into a 1.1 ml analytical vial.

UHPLC-DAD analytical method

Compared to the previously optimized procedures [11, 24] a more selective chromatographic method has been developed for the determination of seed coating insecticides in single bees. UHPLC-DAD instrumental conditions were: eluent flow rate of 0.4 mL min-1, binary water/acetonitrile gradient elution (eluent A: water/acetonitrile 90:10; eluent B: acetonitrile; 0-3.5 min: 100% eluent A; 3.5-14 min: linear gradient from 0 to 12.7% of eluent B; 14-14.5 min: linear gradient to 66.7% of eluent B; 14.5-17.5 min: 66.7% of eluent B; 17.5-18 min: linear gradient to 100% of eluent B; 18-20 min: 100% eluent B), 5 µL of injector volume, 45 °C of column temperature. Detector signal at λ=278 nm for fipronil, λ=252 nm for thiamethoxam and λ=269 nm for clothianidin and imidacloprid were adopted for analytes quantification. Thiacloprid and acetamiprid, neonicotinoids that are not used for corn seed coating in Europe, can also be quantified (λ=244 nm) by the present procedure along with N-desmethyl thiamethoxam (λ=272 nm) a well known thiamethoxam degradation product [50]. The external instrumental calibration was performed by analysis of 50-1000 µg L-1 standard solutions of each insecticide in 50% water-methanol, on a daily basis.

UHPLC/Q-TOF-MS analytical method

UHPLC/Q-TOF-MS analysis used identical elution conditions previously optimized for the UHPLC-DAD method. The Q-TOF mass spectrometer operated in the positive ESI mode for the detection of thiamethoxam, clothianidin, imidacloprid, N-desmethyl thiamethoxam, acetamiprid and thiacloprid and in the negative ESI mode for fipronil (ionization mode switching at 17.5 min). Full scan mass spectra were recorded as centroid over the range 50–1000 m/z with a scan rate of 2 spectra/s. Q-TOF calibration was daily performed with the manufacturer's solution. For all chromatographic runs the m/z 391.28429 relative to the diisooctyl phthalate molecular ion, always present as impurity residue, was set as lock mass for accurate mass analysis. The instrument provided a typical resolving power (FWHM) of 18000 (m/z 922.0098). Mass spectra acquisition and data analysis was processed with Masshunter B.04.00 software (Agilent). External instrumental calibration was performed by analysis of 2-500 ng/bee matrix-matched standard solutions of each insecticide (blank samples fortificated after the final filtration step of the optimized extraction procedure). Quantification was made on the basis of peak area from extracted ion current profiles of the respective [M+H]+ and [M-H]- (fipronil) ions with a mass window of 0.05 Da.

Results and discussion

Although rapid methodologies for the analysis of neonicotinoid insecticides in environmental matrices of interest in the study of colony loss phenomena (i.e. guttation drops and particulate matter) have been recently optimized in our laboratory [11, 24], the direct UHPLC-DAD analysis of methanol or acetonitrile extracts obtained from exposed bees showed some drawbacks, mainly in terms of chromatographic interferences and residues precipitation (probably waxes) [11, 33, 34,]. On the other hand, specific procedures combining solvent extraction, clean-up and LC-MS analysis are quite elaborate and time consuming, but they undoubtedly guarantee high levels of accuracy and sensitivity that make the analysis of bees exposed at very low levels of insecticides possible. The analytical method proposed in the present work, coupling the advantages of a simplified sample preparation method (QuEChERS) with the possibility to use a simpler instrumentation (UHPLC-DAD), can be easily applied to the analysis of neonicotinoid insecticides in single bees after acute exposure, as is the case with the direct contamination of flying bees with corn seed coating particles.

Optimization of extraction and clean-up procedure

In order to obtain a satisfying chromatographic selectivity, even at low concentrations, different extraction solvents were tested, i.e. acetone, ethyl ether, dichloromethane, methanol, acetonitrile, and water - in acidic solution (pH=2, by phosphoric acid) too. First attempts confirmed that acetone and ethyl ether give unsatisfactory recovery factors for neonicotinoids (38-78% and 10-20% for acetone and ethyl ether respectively), while dichloromethane showed good recovery factors (74-99%) but severe matrix interferences mainly affecting clothianidin and imidacloprid determination in most samples. On the contrary, water and acidic solutions present matrix interferences affecting the determination of thiamethoxam (the most water-soluble of the neonicotinoids in question). Some of those interfering peaks could be partially removed by liquid-liquid partitioning with n-hexane or CH2Cl2 which in turn significantly lowers the recovery of the analytes (40-94% for n-hexane, 15-25% for CH2Cl2). The best essayed extraction solvents in terms of both recovery factors and cleanliness from chromatographic interferences were methanol (as previously used in our laboratory) and acetonitrile which has the advantage to be usable also in QuEChERS extraction technique.

Consequently, a different sample pre-treatment approach was studied, starting from well-established QuEChERS methods [33, 35, 37, 40, 46, 49, 51] with some improvements and optimizations in order to apply it, for the first time, to the analysis of single insects. As for the extraction step of the procedure, different combinations of MgSO4/NaCl and MgSO4/NaOAc aqueous solutions were tested as proposed by Kamel [33]: in our case the results showed negligible differences in terms of recovery factors but an improvement in terms of interfering peaks using MgSO4/NaOAc solutions (see Experimental for details).
In any event, the resulting acetonitrile extract could not be directly analyzed by UHPLC mainly for the presence of substances which are prone to precipitate in column or (clearly) just after dilution by water before instrumental injection. In this connection, dispersive SPE clean-up using sorbent like PSA or Amberlite XAD-2 provided an easily solution, which was quicker than conventional SPE, and ensured very good results: clear extracts, absence of precipitation and both highly reproducible and clean from interferences chromatograms were obtained using both tested solid phases.
Finally, after the evaporation of the solvent, negligible differences were found using different solutions to dissolve analytes of interest (i.e. water, acidic water solutions, water/methanol or acetonitrile mixtures); thus a water/methanol solution (90:10) was chosen in order to avoid the unwelcomed peak broadening often observed in UHPLC when an acetonitrile excess is injected.

Optimization of the chromatographic conditions

Taking into account our previously developed procedures [11, 24], UHPLC-DAD instrumental conditions were optimized in order to improve the chromatographic separation of the selected insecticides from possible matrix interferences, simultaneously shortening time of analysis. Best results were obtained using a water/acetonitrile gradient elution program, while modifiers like formic acid (0.01-0.1%), ammonium acetate (0.05%) and ammonium formate (0.05%) added to eluents, produce a few interfering peaks in DAD detection partially overlapping thiamethoxam and clothianidin signals in some samples.
With the optimized UHPLC-DAD method, the elution of five neonicotinoid insecticides, and of N-desmethyl thiamethoxam (a thiamethoxam metabolite) and fipronil (a phenylpyrazole insecticide also used in corn seed coating) takes about 20 min. Of course, if only seed coating neonicotinoids (thiamethoxam, clothianidin and imidacloprid) are of

interest, an anticipated column cleaning step (for instance, at 8.5 min: from 6% to 100% of eluent B in 0.5 min) reduces the time of analysis to 12 min.

Method validation

The performances of the UHPLC-DAD method (summarized in Table 1) were assessed through estimation of accuracy (trueness and precision), sensitivity, selectivity, and linear response range. In view of the impossibility to select real samples (single bees) containing identical concentration of insecticides, both precision and recovery factors were estimated by analysis of a homogenized pool of non-exposed, lyophilized and gently powdered - bees spiked with known amounts of analytes: portions of 0.03 g of this homogenized bee sample (corresponding to the weight of a single lyophilized bee) were added with 50-200 ng of all the analytes (at least 4 concentration levels, 2-5 samples for each level) and analyzed by the optimized method. The results (i.e. the slopes of the recovery functions, see Fig. S1) evidenced excellent recovery factors: 94±2 % for thiamethoxam, 97±2 % for clothianidin, 87±4 % for imidacloprid, 83±2 % for thiacloprid, 93±1 % for acetamiprid and 97±2 % for N-desmethyl thiamethoxam. Conversely, unsatisfactory recovery was obtained for fipronil (30±3 %), a phenylpyrazole insecticide also used in corn seed coating, which is a more lipophilic compound than the analyzed neonicotinoids. Precision levels (repeatability), associated to the aforementioned spiked samples, were of about 5% for thiamethoxam, N-desmethyl thiamethoxam and thiacloprid, 7% for clothianidin and imidacloprid and 10% for acetamiprid. As expected, the scarce recovery obtained for fipronil goes with a higher uncertainty (ca 50%). Therefore, the method guarantees both satisfactory recovery factors and good precision levels for each neonicotinoid insecticide, but it shows unsuitable performances for fipronil.

Table 1 Limits of detection (LOD), repeatability (relative standard deviation), and recovery factors of the entire ultra-high-performance liquid chromatography–diode-array detection analytical procedure for the analysis of neonicotinoid insecticides in single bees

	LOD (ng/bee)	Repeatability[a] (%)	Recovery factor[a] (%)
Thiamethoxam	5	5	94±2
Clothianidin	7	7	97±2
Imidacloprid	7	7	87±4
N-Desmethyl thiamethoxam	5	5	97±2
Acetamiprid	11	10	93±1
Thiacloprid	5	5	83±2

a From the analysis of bee samples (powdered) spiked with 50–200 ng of each insecticide per bee

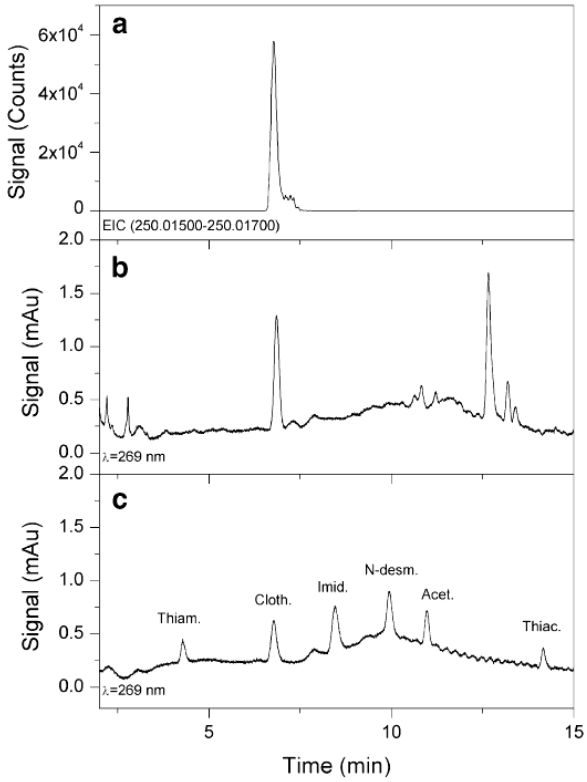

Fig. 1. Ultra-high-performance liquid chromatography (UHPLC)–quadrupole–time of flight mass spectrometry (a) and UHPLC–diodearray detection (b) chromatograms of a real sample, a single bee (exposed in the field to seed coating particulate) containing 165 ng of clothianidin. Chromatogram of a standard solution (200 μgL−1) of each neonicotinoid insecticide (c). *Thiam*. thiamethoxam, *Cloth*. clothianidin, *Imid*. imidacloprid, *N-desm*. N-desmethyl thiamethoxam, *Acet.* acetamiprid, *Thiac.* thiacloprid

The linear response range was experimentally tested for each analyte by instrumental calibration functions up to 100 mg L-1 (r2 > 0.999, p < 10-8). Method sensitivity (the slope of the calibration function) appears to be similar for each neonicotinoid insecticide, if it is related to mass concentration. Again, a lower sensitivity, that will contribute to a higher instrumental limit of detection (LOD), has been observed for fipronil.

The UHPLC-DAD method is characterized by instrumental LOD of ca 8 μg L-1 for thiamethoxam, N-desmethyl thiamethoxam, clothianidin, imidacloprid and acetamiprid (13 μg L-1 for thiacloprid and fipronil), all computed from the parameters of calibration functions using the procedure suggested by IUPAC [52]. In the analysis of real samples by the complete procedure, experimental uncertainties account for actual LOD values of 5 ng/bee for thiamethoxam, N-desmethyl thiamethoxam and thiacloprid, 7 ng/bee for clothianidin and imidacloprid and 11 ng/bee for acetamiprid. As expected, these LOD values are significantly higher than those reported for HPLC-MS methodologies [32-35]. Nevertheless, the UHPLC-DAD method requires a simpler instrumentation, easily fitting in common analytical laboratories, and its LODs are adequate for the analysis of single bees exposed to acute levels of neonicotinoid insecticides.

The combination of optimized QuEChERS, dispersive SPE and UHPLC elution steps efficiently reduces the presence of interfering peaks in the UHPLC-DAD chromatograms of real samples. The absence of chromatographic interferences in the UHPLC-DAD method has further been verified by UHPLC-Q-TOF-MS analysis of both spiked and real samples, using identical chromatographic conditions. Mono-protonated and deprotonated molecular ions, attributable to the single analytes, were always obtained as the main peaks for each insecticide in both standard solutions, spiked and real samples. Negligible amounts of sodiated molecular ions were evidenced in both spiked and real samples while there were no-traces of potassiated and ammoniated adduct ions. Analysis of the extracted ion profiles revealed the presence of a small shoulder at retention time higher than the main peak for each insecticide. More selective extracted ion performed even at 0.002 Da suggested that these shoulders could be attributable to isomeric forms of these analytes (except for acetamiprid, whose shoulder presents traces of some interfering species). Anyway, the effect of these isomers on the UHPLC-DAD peaks appear to be very limited (Figure 1).

Table 2 Concentrations of neonicotinoid insecticides in bees (ng/bee) obtained by the optimized procedure using two independent detection systems: diode-array detection (DAD) and quadrupole–time-of flight mass spectrometry (Q-TOF-MS)

	Thiamethoxam		Clothianidin		Imidacloprid		*N*-Desmethyl thiamethoxam		Acetamiprid		Thiacloprid	
	DAD	Q-TOF-MS	DAD	Q-TOF-MS	DAD	Q-TOF-MS	DAD	Q-TOF-MS	DAD	Q-TOF-MS	DAD	Q-TOF-MS
Bee powder spiked with about 50 ng of each neonicotinoid per bee[a]	49.5±0.3	54±9	54±3	47±4	45±1	43±1	53.2±0.1	48±2	62±3	60±2	47.9±0.4	57±2
Bee powder spiked with about 100 ng of each neonicotinoid per bee[a]	83±7	87±7	97±8	96±9	74±7	70±4	94±7	91±6	98±4	93±4	81±6	79±6
Single bee, exposed in the field to thiamethoxam[b]	74.9	79.3										
	13.4	13.9										
	6.3	5.6										
Single bee, exposed in the field to clothianidin[b]			1,580	1,560								
			2,250	2,200								
			1,220	1,160								
			580	610								
			165	158								
Single bee, exposed in the field to imidacloprid[b]					9.4	9.1						
					13.6	13.5						

aAverage value and standard deviation of two independent measurements
b In the analysis of single bees, the estimated uncertainties are 5 % for thiamethoxam and 7 % for both clothanidin and imidacloprid (see the text)

UHPLC-Q-TOF-MS analysis of real samples made a comparison of results between two independent instrumental procedures possible. Results from spiked samples (n=6, for each neonicotinoid quantified by both detection techniques) were compared by paired t-

test, obtaining non significant differences between mean concentrations measured by the two procedures (α=0.05/2, *p* values of 0.66, 0.25, 0.98, 0.20, 0.053 and 0.09 for thiamethoxam, clothianidin, imidacloprid, N-desmethyl thiamethoxam, acetamiprid and thiacloprid, respectively). Also the analyses of single bees (from field exposure tests, n=10, results in Table 2) evidenced no significant difference in the experimentally measured concentrations (p values of the paired t-test were always higher than 0.25). Our results indicate the possible absence of interferences for the optimized UHPLC-DAD method with a satisfactory accuracy in the analysis of single bees exposed to neonicotinoid insecticides.

Analysis of real samples

The method is currently applied to the analysis of honeybees in both field and laboratory studies aimed to better clarify main exposure routes and real toxicity of these insecticides. Some preliminary results are here provided. First, bees collected in the field after direct exposure to seed coating particulate during the corn sowing always show high levels of insecticides that confirm both our previous observation [11, 25, 26] and the relevance of this exposure-uptake mechanism in the severe colony loss phenomena observed by beekeepers in spring. For instance, in recent sowing experiments using corn seeds coated with clothianidin (Poncho, 1.25 mg/seed, see ref. 26 for details), short time exposure of caged bees to particulate matter emitted by the drilling machine (ca 30 s, simulating 1-2 flights across the sowing field) gave rise an effective contamination of 165-2250 ng/bee of insecticide; these lethal concentrations agree with the levels measured in foraging bees found dead at beehive immediately after the sowing [11, 21].

Another current study in which our analytical method has been successfully applied deal with degradation mechanisms of neonicotinoids after bee uptake. In this respect, it is worth noticing that, in the past, spring mortality was often hard to associate with neonicotinoid contamination, mainly because analysis of bees found dead in the field or close the hive exhibited very low concentrations of these insecticides (see for instance the bee deaths occurred in Italy in Spring 2008 [53]). As is commonly the case, the sampling-analysis procedure was carried out some days after the bees' death, our hypothesis was that a metabolic degradation of the insecticide could significantly affect its real concentration. First laboratory tests (bees were administered with 250-500 ng/bee of thiamethoxam, in alcoholic solutions or adsorbed in talc particles, deposed on the bee tegument) showed a real degradation, which was more rapid when the bees were alive but

to significant extent also post-mortem. Thanks to the present analytical method we were able to carry out experiments at lower doses (60-125 ng/bee of thiamethoxam, that approach LD50 by contact [9, 54]). We found that bees died within 24 h after administering 125 and 60 ng/bee of thiamethoxam, contain 22-67 and 29-38 ng/bee respectively (with lower concentrations if the analysis is delayed); but survived bees seem to contain a time depending decreasing concentration of thiamethoxam, which approach our detection limit (5 ng/bee) in ca 24 h. Therefore, the degradation of the insecticide well documented for sub-lethal doses in Suchail *et al.* [55] is present also in bees exposed to lethal doses.

Currently corn seeds coated with neonicotinoid insecticides are banned in Italy, but these compounds are admitted in spray treatments and, precisely in 2012, also in granular form for soil treatments (i.e. Santana, containing clothianidin). A sowing experiment (spring 2012) using non coated seeds and Santana under normal sowing conditions indicated that a neglecting amount of particles containing the insecticides are released in atmosphere. Indeed, during sowing, the concentration of clothianidin in PTS sampled at the field margin (and in bees collected in the field too) was always below the LOD of the UHPLC-DAD method.

Conclusions

The analytical method optimized and validated in the present work, based on QuEChERS extraction and clean-up and on UHPLC-DAD instrumental analysis, made the accurate determination of neonicotinoid insecticides in single bees possible and can be easily applied in studies regarding the bee loss phenomena consequent to acute exposure of honeybees to these insecticides in open field. Its main advantage is represented by the capability to evaluate the uptake variability of individual exposed bees, an important parameter in the assessment of both real exposure and its consequent toxic effects [13]. As a matter of fact, the method is currently applied in the quantification of new exposure mechanisms of honeybees to neonicotinoid insecticides and in the study of their degradation processes, both in vivo and post mortem. In this connection, new evidences on the rapid metabolic pathway which takes places in bees after acute exposure to these insecticides, could explain the remarkable lack of insecticides often detected in bees collected in the field some days after their death.

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Conclusions

Conclusions

Guttation, neonicotinoids and the problems of eco-toxicity

The presence of raised levels of insecticide in guttation droplets of maize seedlings, varying from 10 ppm up to 1000 ppm, with an average of 250 ppm, is the first demonstration of the existence of potentially lethal sources for the acute poisoning of bees, tied to the sowing of coated maize seed; (the risk is, obviously, extended to other pollinators (Goulson *et al.*, 2008; Brittain and Potts, 2011; Blacquière *et al.*, 2012; Gill *et al.*, 2012), in particular hymenoptera of the genus *Andrena* spp., which nest and winter in the soil and re-commence activity in the Spring at the moment when guttation are produced (Hardstone and Scott, 2010; Tuell and Issacs, 2010). The phenomenon is not strictly tied to sowing of maize with pneumatic seed drills. The quantity of insecticide detected is comparable to that used in the common leaf treatments with identical active ingredients and certainly sufficient to kill bees which come into contact with them (Girolami *et al.*, 2009; Tapparo *et al.*, 2011), whether by ingestion or topically. Large variations in the concentration of active ingredient in guttation were observed, and an identification of the cause was sought. The results, similar for the various neonicotinoidsunder consideration, (Apenet, 2010) suggested, in the context of a progressive reduction of concentrations over time, limiting the examination to the early weeks does not show conclusive results (in influencing the concentrations). Other factors were considered such as 1) the density of the sowing; 2) differing watering regimes; 3) the texture of the soil; 4) differences between individual plants; 5) the time of day. Of the variables under consideration, the time of day of the collection proved to be the factor which most determined the concentration, but not unequivocally so, with the rule that as the daylight proceeded so the concentration increased (although it sometimes showed a reversal of the result. Subsequently it could be shown that the time of the collection could be conditioned by the fall of the droplets themselves, whereby evaporation increased the concentration until the droplets fell. Consequently, droplets just formed showed very low concentrations for a brief period. Unpublished data, obtained during the mechanical shaking of the plants at 15 minute intervals (to simulate windy conditions) showed that, in conditions of high relative humidity, there were no differences between the shaken plants and those unshaken; whilst in plants which were kept in conditions of low humidity, the concentrations were markedly higher due to phenomena of evaporation (in unshaken

plants). In inducing a frequent fall of the droplets, the concentration (even in dry air conditions) showed results similar to those obtained in saturation humidity. As a synthesis, the raised levels of active ingredient in guttation drops are due, above all to the evaporation of the liquid and the consequent concentration. This phenomenon does not occur if continuous gusts of strong wind cause the forming drops to fall. Consequently it can be hypothesised that a light fresh wind increases the concentration whilst a sudden strong wind causes the concentration to decrease. The data, although clear, is not yet sufficient for publication, and requires further replication and verification (Apenet, 2010).

Acute poisoning of bees by particles emitted by seed drills

Chemico-physical aspects of the particulates

The particles emitted into the air proved to be different as regards quantity and also, little by little,of different dimensions the further they got from the seed drill. Apart from the coarser particles produced (20-200µm), significant quantities of micro and sub-microscopic particles (0.5-10 µm) showed greatly increased mobility in the atmosphere (Tapparo *et al.*, 2012/a). The insecticide content of the particles was shown on chemical analysis to be above 20% (Girolami *et al.*, 2011). The data confirmed the possibility that bees are subject to acute poisoning during flights if contaminated with particles of seed coating projected into the atmosphere.

Particulates and the mode of acute poisoning of bees

Though it is officially maintained that the path of the poisoning of bees is contact with insecticide powder falling on the land simply through the contamination of pollen and nectar (given the systemic nature of the molecules examined), the hypothesis proved not very credible. No acute poisoning of bees was observed in bees which came into contact with dew or guttation (of the spontaneous vegetation at the margins of the plots) contaminated by particles emitted from the drills (Marzaro *et al.*, 2011). This was further confirmed by chemical analysis. The hypothesis that lethal poisoning occurred in flight by direct contact with the toxic cloud containing fragments of seed shell blown into the atmosphere by pneumatic seed drills was proven valid. It was also confirmed by appropriate biological tests and chemical analysis (Girolami et al, 2012; Tapparo *et al.*, 2012/a) opportunely put in place, and capable of efficiently analysing a single bee. (Tapparo *et al.*, 2012/b). In particular, it was demonstrated that bees flying free in the vicinity of a seed drill in action could take on doses of largely lethal insecticide (for

clothianidin doses greater than 600 ng/bee, whilst imidacloprid exceeded 4000 ng), an amount some hundreds of times greater than LD_{50}. These quantities are probably due to the characteristics of the bees' furry tegument which is designed for the harvesting of pollen. Moreover, it was proved that high humidity after contamination increased the toxic effect of the particles presumably by increasing their adherence to the tegument.

The poisoning of bees was demonstrated using a seed drill in the habitual foraging path of bees.

Other tests envisaged the rapid passages of caged bees in the proximity of the seed drill at progressive distances from the drill and at differing heights. It was shown that a single pass (at a fast walking pace) within the first 10 metres of the drill, as a rule, caused lethal poisoning. Chemical analysis of individual bees showed enormous quantities of active ingredient, whether dead in the laboratory, or gathered from in front of the hives. It was demonstrated that, in the absence of a sustained wind, a toxic cloud formed, elliptical in shape, some 20 metres in diameter and at least 3 metres high.

Another aspect of particular importance is the opportunity to explain how a bee, though killed by insecticide, can show a negative result (to the presence of active ingredient) in chemical analysis (Bortolotti *et al.*, 2009). Taking account of the fact that the samples of dead bees collected from in front of the hives are taken, for analysis, to the veterinary services some 24-48 hours after the widespread deaths are observed by the beekeepers; the explanation may be relatively simple given the possible leaching of particulates of systemic insecticide from moribund bees due to the action of dew or rain. The earlier enquiries, contrary to the first hypotheses, tended to exclude enzyme degeneration. The attempt to prove that dead bees cannot possibly contain relevant levels of insecticide attributable to the use of coated seedwould constitute the last piece in the puzzle of the widespread Spring deaths of bees.

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