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Neonicotinoids, bee disorders and the sustainability of pollinator services

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In less than 20 years, neonicotinoids have become the most widely used class of insecticides with a global market share of more than 25%. For pollinators, this has transformed the agrochemical landscape. These chemicals mimic the acetylcholine neurotransmitter and are highly neurotoxic to insects. Their systemic mode of action inside plants means phloemic and xylemic transport that results in translocation to pollen and nectar. Their wide application, persistence in soil and water and potential for uptake by succeeding crops and wild plants make neonicotinoids bioavailable to pollinators at sublethal concentrations for most of the year. This results in the frequent presence of neonicotinoids in honeybee hives. At field realistic doses, neonicotinoids cause a wide range of adverse sublethal effects in honeybee and bumblebee colonies, affecting colony performance through impairment of foraging success, brood and larval development, memory and learning, damage to the central nervous system, susceptibility to diseases, hive hygiene etc. Neonicotinoids exhibit a toxicity that can be amplified by various other agrochemicals and they synergistically reinforce infectious agents such as *Nosema ceranae* which together can produce colony collapse. The limited available data suggest that they are likely to exhibit similar toxicity to virtually all other wild insect pollinators. The worldwide production of neonicotinoids is still increasing. Therefore a transition to pollinator-friendly alternatives to neonicotinoids is urgently needed for the sake of the sustainability of pollinator ecosystem services.

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Introduction

The introduction to the market in the early 1990s of imidacloprid and thiacloprid opened the neonicotinoid era of insect pest control [1]. Acting systemically, this new class of neurotoxic insecticides is taken up by plants, primarily through the roots, and translocates to all parts of the plant through xylemic and phloemic transport [2]. This systemic property combined with very high toxicity to insects enabled formulating neonicotinoids for soil treatment and seed coating with typical doses from 10 to 200 g ha^{-1} high enough to provide long lasting protection of the whole plant from pest insects.

Neonicotinoids interact with the nicotinic acetylcholine receptors (nAChRs) of the insect central nervous system. They act mainly agonistically on nAChRs on the postsynaptic membrane, mimicking the natural neurotransmitter acetylcholine by binding with high affinity [3–5,6••,7•,8••]. This induces a neuronal hyper-excitation, which can lead to the insect's death within minutes [6,9]. Some of the major metabolites of neonicotinoids are equally neurotoxic, acting on the same receptors [10–12] thereby prolonging the effectiveness as systemic insecticide. The nAChR binding sites in the vertebrate nervous system are different from those in insects, and in general they have lower numbers of nicotinic receptors with high affinity to neonicotinoids, which are the reasons that neonicotinoids show selective toxicity for insects over vertebrates [9,13].

The main neonicotinoids presently on the market are imidacloprid, thiamethoxam, clothianidin, thiacloprid, dinotefuran, acetamiprid, nitenpyram and sulfoxaflor [12,14,15]. Since their introduction, neonicotinoids have grown to become the most widely used and fastest

growing class of insecticides with a 2010 global market share of 26% of the insecticide market [16] and imidacloprid the second most widely used (2008) agrochemical in the world [17]. The worldwide production of neonicotinoids is still increasing [18]. Large-scale use in Europe and US started around 2004. Neonicotinoids are nowadays authorised in more than 120 countries for more than 1000 uses [19] for the treatments of a wide range of plants including potato, rice, maize, sugar beets, cereals, oil rapeseed, sunflower, fruit, vegetables, soy, ornamental plants, tree nursery, seeds for export, and cotton.

When used as a seed coating, only 1.6–20% of the amount of active substance applied actually enters the crop to protect it [20], and the remaining 80–98.4% pollutes the environment without any intended action to plant pests. Diffusion and transformation of pesticides in the environment lead to various environmental concentrations and bioavailability, all strongly dependent on the properties of the substance [21]. Because of their high leaching potential, neonicotinoids tend to contaminate surface water and ground water [22–25]. Owing to sorption to organic matter in soil and sediments [24,26], the equilibrium partitioning over soil and water varies with soil type and is typically 1:3 ($\log P = 0.57$) [25]. In countries where monitoring data are available, high levels of neonicotinoid pollution in surface water have been reported [27–30]. In the Netherlands, 45% of 9037 water samples taken from 801 different locations in a nation-wide routine water quality monitoring scheme, over the period 1998 and 2003–2009, exceeded the 13 ng l^{-1} imidacloprid water quality standard, the median concentration being 80 ng l^{-1} and the maximum concentration found being 320 μg l^{-1}, which is acutely toxic to honeybees [27]. In the US, neonicotinoids were also found in surface water. In 108 water samples collected in 2005 from playa wetlands on the Southern High Plains, thiamethoxam was found at an average concentration of 3.6 μg l^{-1} and acetamiprid at 2.2 μg l^{-1} [30].

Neonicotinoids and their metabolites are highly persistent in soil, aquatic sediments and water. To give an example: Six years after a single soil drench application of imidacloprid, residue levels up to 19 μg kg^{-1} could be recovered in *Rhododendron* shrub blossoms [31]. Clothianidin has a half-life in soil between 148–6900 days [32], and imidacloprid 40–997 days [33]. Consequently, neonicotinoids exhibit a potential for accumulation in soil following repeated applications [23] and can be taken up by succeeding crops up to at least two years after application [34]. Imidacloprid has been detected in 97% of 33 soil samples from untreated fields on which treated corn seeds were used 1 or 2 years before the sampling [34]. Concentrations in these soil samples ranged from 1.2 to 22 μg kg^{-1} [34]. Several studies recovered neonicotinoids in wild flowers near treated fields [35,36••]. However, it remains a knowledge gap to what extent the presence in wild flowers results from systemic uptake from polluted soil and water or from direct contamination of the flowers by contaminated dust from seed drilling.

At their introduction, neonicotinoids were assumed to be more efficient than the organophosphates and carbamates that they replaced [37]. As a seed treatment, they could be used in much lower quantities and they promised to be less polluting to the environment. It is however not the quantity that is relevant but the potency to cause harm, which results from toxicity, persistence and bioavailability to non-target species. Indeed, soon after the introduction of neonicotinoids, exposure to its residues in pollen, nectar, sowing dust etc., of non-target pollinating insects became clear. This led to various harmful effects [10,37,38,39••,40,41,42••,43••].

Ecosystem services of pollinators

Amongst the wide diversity of pollinating species [44], bees are the most important. Although bee research mostly focuses on the domesticated *Apis mellifera*, over 25,000 different bee species have been identified (FAO: Pollination; URL: http://www.fao.org/agriculture/crops/core-themes/theme/biodiversity/pollination/en/). Bees provide a vital ecosystem service, playing a key role in the maintenance of biodiversity and in food and fibre production [45–47,48••,49–51]. Pollination comprises an integrated system of interactions that links earth's vegetation, wildlife and human welfare [52]. Of all flowering plants on earth, 87.5% benefits from animal pollination [53]. Globally, 87 of the leading food crops (accounting for 35% of the world food production volume) depend on animal pollination [45]. Pollinator mediated crops are of key importance in providing essential nutrients in the human food supply [54•]. The history of apiculture goes back to pre-agricultural times [55,56] and later co-developed with agriculture [57,58]. In addition, wild bees deliver a substantial and often unappreciated portion of pollination services to agriculture and wildflowers [59,60]. Bees and apiary products have a pharmacological [61,62], scientific and technological [63], poetic [64], aesthetic (springs filled with buzzing bumblebees) culinary (e.g., keeping alive traditional cuisine of patisseries with honey) and cultural value.

Global pollinator decline and emerging bee disorders

Long-term declines have been observed in wild bee populations around the world [47,65–70]. Over the past decades, a global trend of increasing honeybee disorders and colony losses has emerged [71–77]. Winter mortality of entire honeybee colonies has risen in many parts of the world [72•,73,74,75•]. When neonicotinoids were first used, beekeepers started describing different disorders and signs ranging from: bees not returning to the hive, disoriented bees, bees gathered close together in small groups on the ground, abnormal foraging behaviour, the

occurrence of massive bee losses in spring, queen losses, increased sensitivity to diseases and colony disappearance [38,40–43,77]. None of these individual signs is a unique effect of neonicotinoids, other causal factors or other agrochemicals could produce similar signs, which complicates the establishment of a causal link.

Scientific research appears to indicate no single cause explaining the increase in winter colony losses. All viruses and other pathogens that have been linked to colony collapse have been found to be present year-round also in healthy colonies [78]. That colonies remain healthy despite the presence of these infectious agents, supports the theory that colony collapse may be caused by factors working in combination. Farooqui [79•] has analysed the different hypotheses provided by science when searching for an explanation of Colony Collapse Disorder (CCD). Research points in the direction of a combination of reciprocally enhancing causes. Among those, the advance of neonicotinoid insecticides has gained more weight in light of the latest independent scientific results [80,81••,82••]. In the present article, we synthesise the state of knowledge on the role of neonicotinoids in pollinator decline and emerging bee disorders.

Multiple ways of exposure

Neonicotinoids are authorised for a wide range of agricultural and horticultural plants that flower at different times of the year. The systemic properties of neonicotinoids imply translocation to pollen, nectar, and guttation droplets [34,37,83,84]. The persistency and potential contamination of wild plants and trees surrounding the treated crops [36] and the possibility for travelling far outside the fields via surface and ground water [27] and the potential to contaminate wild plants and crops that take up polluted water, means that pollinating insects are likely to be exposed for much of the year to multiple sources of multiple neonicotinoids in their foraging area, but often at very low doses.

Honeybees' exposure to neonicotinoids can occur through ingestion, contact and inhalation (aerosols). Many possible exposure pathways can exist [85•]. Here, we aggregate exposure pathways into: first, intake of food that contain residues; second, nesting material (resin, wax etc.); third, direct contact with spray drift and dust drift during application; fourth, contact with contaminated plants, soil, water; fifth, use of cooling water in the hive; and sixth, inhalation of contaminated air. For bumble bees and other wild bees that nest in soil, contact with contaminated soil is an additional pathway of concern. Leafcutter bees use cut leaf fragments to form nest cells and can thus be exposed to residues in leaves. There are many other conceivable exposure routes, for instance, a bee hive could have been made from timber from trees treated with neonicotinoids and may thus contain residues. However, the best researched exposure pathway is via intake of food. Food with residues can be subdivided into self-collected raw food (nectar, pollen, water, honeydew, extrafloral nectar, guttation droplets, various other edible substances available in the foraging area etc.), in-hive processed food (honey, beebread, royal jelly, wax etc.), and food supplied by bee keepers (high fructose corn syrup, sugar water, sugar dough, bee candy, pollen, pollen substitutes based on soybean flower and other vegetable protein supplements etc.).

Given the large numbers of crops in which neonicotinoids are used and the large scale of use, there is a huge variability in space and time for each possible exposure pathway as well as in their relative importance for the overall exposure at a given place and time. This is further complicated by the fact that the foraging area of a honeybee colony can extend to a radius of up to 9 km around the hive which is never a homogenous landscape [86]. Additionally, suburban areas have become a stronghold for some wild bee species due to the abundance of floral resources in gardens and parks [87]. Thus, bees may be exposed to systemic insecticides which are widely used on garden flowers, vegetables, ornamental trees, and lawns. The relative importance of exposure pathways will also vary according to bee species as they have different foraging ranges, phenologies, and flight times in a day. This can be exemplified by *Osmia* bees in corn growing areas for which intake of guttation droplets may be more important than for honeybees.

Different categories of honeybees could be exposed in different ways and to varying extents [42]. For example, pollen foragers (which differ from nectar foragers) do not consume pollen, merely bringing it to the hive. The pollen is consumed by nurse bees and to a lesser extent by larvae which are thus the ones that are exposed to residues of neonicotinoids and their metabolites [88]. The exposure of nectar foragers to residues of neonicotinoids and metabolites in the nectar they gather can vary depending on the resources available in the hive environment. In addition, foragers take some honey from the hive before they leave for foraging. Depending on the distance from the hive where they forage, the honeybees are obliged to consume more or less of the nectar/honey taken from the hive and/or of the nectar collected, for energy for flying and foraging. They can therefore ingest more or less neonicotinoid residues, depending on the foraging environment [42]. Oral uptake is estimated to be highest for forager honeybees, winter honeybees and larvae [85].

Little is known about the real exposure to contaminated food for different categories of honeybees in a colony, either in terms of contact with pollen or contact with, and possible consumption of, nectar if needed. For wild bees very few data exist on exposure in the field. The amount that wild bees actually consume in the field has not been

measured. EFSA estimated that worker bees, queens and larvae of bumblebees and adult females and larvae of solitary bees are likely to have the highest oral uptake of residues [85].

In 2002, 69% of pollen samples collected by honeybees at various places in France contained residues of imidacloprid and its metabolites [89]. In a systematic sampling scheme covering 5 locations over 3 years, imidacloprid was found in 40.5% of the pollen samples and in 21.8% of the honey samples [90,91]. On the basis of data from authorisation authorities, neonicotinoid residues in nectar and pollen of treated crop plants are estimated to be in the range of below analytical detection limit (0.3 $\mu g\ kg^{-1}$) to 5.4 $\mu g\ kg^{-1}$ in nectar, the highest value corresponding to clothianidin in oilseed rape nectar, and a range of below detection limit (0.3 $\mu g\ kg^{-1}$) to 51 $\mu g\ kg^{-1}$ in pollen, the highest value corresponding to thiamethoxam in alfalfa pollen [85]. A recent review reports wider ranges for pollen: 0.2–912 $\mu g\ kg^{-1}$ for imidacloprid and 1.0–115 $\mu g\ kg^{-1}$ for thiacloprid [92]. Residues of imidacloprid, dinotefuran, and thiamethoxam plus metabolites in pumpkin treated with United States label rates reach average levels up to 122 $\mu g\ kg^{-1}$ in pollen and 17.6 $\mu g\ kg^{-1}$ in nectar [93]. Up to 346 $mg\ l^{-1}$ for imidacloprid and 146 $mg\ l^{-1}$ for thiamethoxam and 102 $mg\ l^{-1}$ clothianidin and have been found in guttation drops from leaves of plants germinated from neonicotinoid-coated seeds [84,94]. In melon, guttation levels up to 4.1 $mg\ l^{-1}$ imidacloprid were found 3 days after a top (US) label rate soil application [95]. In a US wide survey of pesticide residues in beeswax, pollen and honeybees during the 2007–2008 growing seasons, high levels of neonicotinoids were found in pollen (included in [92]) but imidacloprid was also found up to 13.6 $\mu g\ kg^{-1}$ in wax [96]. In Spain, neonicotinoids were found in beeswax samples from apiaries near fruit orchards: 11 out of 30 samples tested positive in ranges from 11 $\mu g\ kg^{-1}$ (acetamiprid) to 153 $\mu g\ kg^{-1}$ (thiacloprid) [97].

Little is known on the presence of neonicotinoids in honeydew. Given differences in life span of aphids and bees, concentrations in plant sap too low to kill aphids could translocate to honeydew and could still produce sublethal effects and chronic toxicity mortality in bees and bee colonies.

Acute and chronic effects of lethal and sublethal exposure

Pesticides can produce four types of effects on honeybees: lethal effects and sublethal effects from acute or chronic exposures.

Acute toxicity is expressed as the lethal dose (LD) at which 50% of the exposed honeybees die within 48 hours: abbreviated to 'LD50 (48 hours)'. Neonicotinoids are highly toxic (in the range of ng/bee) to honeybees [98], both when administered orally and by contact. They also have high acute toxicity to all other bee species so far tested, including various *Bombus* species, *Osmia lignaria* and *Megachile rotundata* [99–102]. *O. lignaria* is more sensitive to both clothianidin and imidacloprid than is *B. impatiens*, with *M. rotundata* more sensitive still [100]. In an acute toxicity test under semi field conditions on the Indian honeybee *Apis cerana indica*, clothianidin showed the highest toxicity, followed by imidacloprid and thiamethoxam [103].

For mass-dying of bees in spring nearby and during sowing of corn seeds coated with neonicotinoids there now is a one to one proven causal link with acute intoxication though contact with the dust cloud around the pneumatic sowing machines during foraging flights to adjacent forests (providing honeydew) or nearby flowering fields [104••,105–109]. Such mass colony losses during corn sowing have also been documented in Italy, Germany, Austria and Slovenia [110,111,104••]. In response to the incidents, the adherence of the seed coating has been improved owing to better regulations, and an improved sowing-technique has recently become compulsory throughout Europe, [112]. Despite the deployment of air deflectors in the drilling machines or improved seed coating techniques, emissions are still substantial and the dust cloud is still acutely toxic to bees [105,109,111,113–115]. Acute lethal effects of neonicotinoids dispersed as particulate matter in the air seem to be promoted by high environmental humidity which accelerates mortality [105]. Honeybees also bring the toxic dust particles they gather on their body into the hive [106]. Sunny and warm days also seem to favour the dispersal of active substances [35].

Lethal effects from chronic exposure refer to honeybee mortality that occurs after prolonged exposure. In contrast to acute lethal effects, there are no standardised protocols for measuring chronic lethal effects. Therefore, in traditional risk assessment of pesticides they are usually expressed in three ways: LD50: the dose at which 50% of the exposed honeybees die (often, but not always, within 10 days); NOEC (No Observed Effect Concentration): the highest concentration of imidacloprid producing no observed effect; and LOEC (Lowest Observed Effect Concentration): the lowest concentration of imidacloprid producing an observed effect. However, for neonicotinoids and its neurotoxic metabolites, lethal toxicity can increase up to 100,000 times compared to acute toxicity when the exposure is extended in time [10]. There has been some controversy on the findings of that study, which is discussed in detail by Maxim and Van der Sluijs [40,42]. However, the key finding that exposure time amplifies the toxicity of neonicotinoids is consistent with later findings. Micro-colonies of bumblebees fed with imidacloprid showed the same phenomenon [102]: at one tenth of the concentration of the toxin in feed, it took twice as long to produce 100% mortality in a

bumblebee microcolony. At a 100 times lower dose, it took ca. four times longer to produce 100% mortality. The measurable shortening of the life span ceases to occur only when a dose was administered, for which the (extrapolated) chronic intoxication time would be longer than the natural life span of a worker bumblebee. This implies that the standard 10 day chronic toxicity test for bees is far too short for testing neonicotinoids. Indeed, honeybees fed with one tenth of the LC50 of thiamethoxam showed a 41.2% reduction of life span [116]. Recent studies have shown that chronic toxicity of neonicotinoids can more adequately be expressed by time to 50% mortality instead of by the 10 day LD50 [117–120,121•,122]. There is a linear relation between log daily dose and log time to 50% mortality [118,120,121•]. In experiments with honeybee colonies, similar long term chronic effects have indeed been found with typical times of 14–23 weeks to collapse 25–100% of the colonies exposed to imidacloprid-contaminated food at 20 $\mu g\ kg^{-1}$ [123] and 80–120 days for 1 $mg\ kg^{-1}$ dinotefuran and 400 $\mu g\ kg^{-1}$ clothianidin [76]. Note that these studies used concentrations that are on the high end of the currently reported ranges of concentrations found in the field. However, such data are sparse and limited to a few crops, so it cannot yet be concluded whether such concentrations are rare or common in the field.

At low concentrations of neonicotinoids, sublethal effects can occur. Sublethal effects involve modifications of honeybee behaviour and physiology (e.g., immune system). They do not directly cause the death of the individual or the collapse of the colony but may become lethal in time and/or may make the colony more sensitive (e.g., more prone to diseases), which may contribute to its collapse. For instance, an individual with memory, orientation or physiological impairments might fail to return to its hive, dying from hunger or cold. This would not be detected in standard pesticide tests, which focus on acute mortality. A distinction can be made between acute and chronic sublethal effects. Acute sublethal effects are assessed by exposing bees only once to the substance (by ingestion or by contact), and observing them for some time (variable from one laboratory to another, from several minutes to four days). Chronic sublethal effects are assessed by exposing honeybees more than once to neonicotinoids during an extended period of time (e.g., every 24 hours, for 10 days). Both acute and chronic sublethal effects are expressed as NOEC and/or LOEC (No or Lowest Observable Effect Concentration, respectively) [42].

In an extensive review Desneux *et al.* found that sublethal effects of neonicotinoids exist on neurophysiology, larval development, moulting, adult longevity, immunology, fecundity, sex ratio, mobility, navigation and orientation, feeding behaviour, oviposition behaviour, and learning [124]. All these effects have been reported for pollinators and all have the potential to produce colony level, population level and community level impacts on pollinators.

At field realistic concentrations (1 $\mu g\ l^{-1}$) imidacloprid repels pollinating beetles while at concentrations well below the analytical detection limit (0.01 $\mu g\ l^{-1}$) it repels pollinating flies [125]. This implies that imidacloprid pollution may disrupt pollination both in polluted nature and in agricultural lands. On honeybees, imidacloprid has no repelling effect at field realistic concentrations: it starts being repellent at 500 $\mu g\ l^{-1}$ [126]. In some plant protection formulations, neonicotinoids are mixed with bee repellents. However, the persistence of neonicotinoids exceeds that of the repellence and their systemic properties differ. Besides, if bees are effectively repelled and avoid the contaminated flowers, pollination is disrupted because plants are not visited by bees.

Sublethal doses of neonicotinoids impair the olfactory memory and learning capacity of honeybees [127,128,129•,130] and the orientation and foraging activity [131]. The impact of sublethal exposure on the flying behaviour and navigation capacity has been shown through homing flight tests [82,126,132,133]. Exposed to a very low concentration (0.05 $\mu g\ kg^{-1}$) imidacloprid honeybees show an initial slight increase in travel distance. However, with increasing concentration, starting at 0.5 $\mu g\ kg^{-1}$ imidacloprid decreases distance travelled and interaction time between bees, while time in the food zone increases with concentration [134•]. Imidacloprid disrupts honeybee waggle dancing and sucrose responsiveness at doses of 0.21 and 2.16 $ng\ bee^{-1}$ [135].

If honeybee brood is reared at suboptimal temperatures (the number of adult bees is not sufficient to maintain the optimal temperature level), the new workers will be characterised by reduced longevity and increased susceptibility to pesticides (bee-level effect) [136]. This will again result in a number of adult bees insufficient to maintain the brood at the optimal temperature, which may then lead to chronic colony weakening until collapse (colony-level effect).

Sublethal effects seem to be detected more frequently and at lower concentrations when bumblebees (*Bombus terrestris*) have to travel to gather food, even when the distances are tiny. No observable impacts of imidacloprid at field realistic concentrations on micro-colonies of *B. terrestris* provided with food in the nest were found, but when workers had to walk just 20 cm down a tube to gather food, they exhibited significant sublethal effects on foraging activity, with a median sublethal effect concentration (EC_{50}) of 3.7 $\mu g\ kg^{-1}$ [102]. In queenright bumblebee colonies foraging in a glasshouse where food was 3 m away from their nest, 20 $\mu g\ kg^{-1}$ of imidacloprid caused significant worker mortality, with bees dying at the feeder. Significant mortality was also observed at

10 μg kg^{-1}, but not at 2 μg kg^{-1} [102]. Bumblebees exhibit concentration-dependent sublethal responses (declining feeding rate) to imidacloprid starting at 1 μg l^{-1} in syrup, while honeybees seemed unaffected [137].

Field-relevant concentrations of imidacloprid, used alone or in mixture with λ-cyhalothrin, were shown to impair pollen foraging efficiency in bumblebee colonies [138•]. In an attempt to fulfill colony needs for pollen, more workers were recruited to forage instead of taking care of brood. This seemed to affect brood development resulting in reduced worker production [138•]. Bumblebee colonies have been exposed to field realistic levels of imidacloprid (0.7 μg kg^{-1} in nectar, 6 μg kg^{-1} in pollen) for two weeks in the laboratory. When subsequently placed back in the field and allowed to develop naturally for the following six weeks, treated colonies showed an 85% reduction in queen production and a significantly reduced growth rate [81••]. Effects on bumblebee reproduction occur at imidacloprid concentrations as low as 1 μg l^{-1} [139•] which is highly field-realistic.

It has also been shown that pesticides like imidacloprid act on the hypopharyngeal glands of honeybee nurses by degenerating the tissues [140,141,142••], which induces a shift from nest to field activities. In the native stingless bee *Melipona quadrifasciata anthidioides*, imidacloprid causes impairment of the mushroom bodies which are involved in learning [143]. Imidacloprid and clothianidin have been shown to be potent neuromodulators of the honeybee brain, causing mushroom body neuronal inactivation in honeybees, which affect honeybee cognition and behaviour at concentrations that are encountered by foraging honeybees and within the hive [8]. Sublethal doses of imidacloprid were also found to have cytotoxic activity in the Malpighian tubules in honeybees that make up the excretory and osmoregulatory system [144]. Exposure to thiamethoxam has also been shown to result in morphological impairment of the bee brain and bee midgut [116].

Exposure to neonicotinoid residues leads to a delayed development of honeybee larvae, notably in the early stages (day 4 to day 8) [145]. This can favour the development of the *Varroa destructor* parasitic mite within the colony. Likewise, the life span of adult bees emerging from the exposed brood proved to be shorter.

Short-term and mid-term sublethal effects on individuals or age groups result in long-term effects at the colony level, which follow weeks to months after the exposure, such as honeybee colony depopulation and bumblebee colony queen production [76,81••,123,138•]. As it has recently been acknowledged, the field tests on which the marketing authorisation of the use of neonicotinoids is essentially based were not developed to detect sublethal nor long-term effects on the colony level, and the observation of the performances of colonies after experimental exposure do not last long enough [85]. Major weaknesses of existing field studies are the small size of the colonies, the very small distance between the hives and the treated field and the very low surface of the test field. As a consequence of these weaknesses, the real exposures of the honey bees during these field tests are highly uncertain and may in reality be much smaller than what has been assumed in these field studies. [85]

In addition, the meta-analysis [146•] demonstrates that field tests published until now on which European and North American authorizations are based, lack the statistical power required to detect the reduction in colony performance predicted from the dose–response relationship derived from that meta-analysis. For this purpose, the tests were wrongly designed, there were too few colonies in each test group, and the follow up time monitoring the long term colony level impacts were too short to detect many of the effects described above. Nonetheless, these field studies have been the basis for granting the present market authorizations by national and European safety agencies. The meta-analyses combined data from 14 previous studies, and subsequently demonstrated that, at exposure to field realistic doses, imidacloprid does have significant sublethal effects, even at authorised levels of use, impairs performance and thus weakens honeybee colonies [146•].

A further limitation of field studies is their limited reproducibility due to the high variability in environmental conditions in the foraging area of honeybees, which extends up to a 9 km radius around the hive. Observations made in a particular field experiment might not be representative of the range of effects that could occur in real conditions. Owing to the large variability of factors that cannot be controlled (e.g. other stressors, soil structure, climate, combination of plants attractive to bees etc.), current field experiments only give information about the particular situation in which they were done.

The challenges of field studies became also clear in the debates over the highly contested field study recently conducted by the Food and Environment Research Agency (FERA) which resorts under the UK Department for Environment, Food and Rural Affairs (DEFRA). This study was set up in response to the *Science* publication that showed that a short term exposure of bumblebees to field realistic imidacloprid concentrations causes a long term 85% reduction in queen production [81••]. At three sites 20 bumblebee colonies were exposed to crops grown from untreated, clothianidin-treated or imidacloprid-treated seeds. The agency concluded that 'no clear consistent relationships' between pesticide levels and harm to the insects could be found [FERA: URL: http://www.fera.defra.gov.uk/scienceResearch/scienceCapabilities/chemicalsEnvironment/documents/reportPS2371V4a.pdf].

However, it turned out that the control colonies themselves were contaminated with the pesticides tested [147]. Further, thiamethoxam was detected in two out of the three bee groups tested, even though it was not used in the experiment. The major studies that have measured neonicotinoid residues in pollen collected by honeybees clearly show that neonicotinoids are found in pollen all over the year and in all studied regions, not only after the sowing or during the flowering period [89,91,96]. With the present scale of use, it will be very difficult to find a control site where bees cannot come into contact with neonicotinoids.

Given all the major limitations to the reliability of outcomes of field studies, it is recommendable to give more weight in the risk assessment to reproducible results from controlled lab studies and use the ratio between the environmental concentration and the no effect concentration as the main risk indicator [40,42]. It could perhaps be linked to modelling to explore how, and to what the degree, the various well-known sublethal effects on individual bees can weaken the colony [148].

A key aspect in honeybee biology is that the colony behaves as a 'superorganism' [149]. In a colony, sufficient membership, so that the number of organisms involved in the various tasks to maintain that colony, is critical, not the individual quality of a task performed by an individual bee. Varying between winter and summer, the 10,000–60,000 honeybees that typically form a colony function as a cooperative unit, maintaining intraorganismic homeostasis as well as food storage, nest hygienic, defence of the hive, rearing of brood etc. Hence, sublethal effects affecting the number of individuals that perform specific functions, can influence the functioning of the whole colony. In a simplified theoretical modelling approach, colony failure can be understood in terms of observed principles of honeybee population dynamics [150]. A colony simulation model predicts a critical threshold forager death rate above which rapid population decline is predicted and colony failure is inevitable. High forager death rates draw hive bees towards the foraging population at much younger ages than normal, which acts to accelerate colony failure [150].

Synergistic effects: pesticide–pesticide and pesticide–infectious agents

A synergy occurs when the effect of a combination of stressors is higher than the sum of the effect of each stressor alone. When neonicotinoids are combined with certain fungicides (azoles, such as prochloraz, or anilides, such as metalaxyl) or other agrochemicals that block cytochrome P450 detoxification enzymes, their toxicity increases by factor from 1.52 to 1141 depending on the combination [151,152]. The strongest synergism has been found for triflumizole making thiacloprid 1141 times more acutely toxic to honeybees [151]. This synergistic effect is the subject of patents by agrochemical companies [152,153].

Synergy has also been demonstrated for neonicotinoids and infectious agents. Prolonged exposure to a non-lethal dose of neonicotinoids renders beehives more susceptible to parasites such as *Nosema ceranae* infections [39••,154••, 155•,156]. This can be explained either by an alteration of the immune system or by an impairment of grooming and allogrooming that leads to reduced hygiene at the individual level and in the nest, which gives the pathogens more chances to infect the bees. The same mechanism, where the balance between an insect and its natural enemies is disturbed by sublethal exposures to neonicotinoids that impairs grooming, is well known and often used in pest management of target insects [157–161].

Conclusion and prospects

In less than 20 years, neonicotinoids have become the most widely used class of insecticides. Being used in more than 120 countries in more than 1000 different crops and applications, they now account for at least one quarter of the world insecticide market. For pollinators, this has transformed the agrochemical landscape to one in which most flowering crops and an unknown proportion of wild flowers contain varying concentrations of neonicotinoids in their pollen and nectar. Most neonicotinoids are highly persistent in soil, water and sediments and they accumulate in soil after repeated uses. Severe surface water pollution with neonicotinoids is common. Their systemic mode of action inside plants means phloemic and xylemic transport that results in translocation to pollen and nectar. Their wide application, persistence in soil and water and potential for uptake by succeeding crops and wild plants make neonicotinoids bioavailable to pollinators in sublethal concentrations for most of the year. This results in the frequent presence of neonicotinoids in honeybee hives. Neonicotinoids are highly neurotoxic to honeybees and wild pollinators. Their capacity to cross the ion-impermeable barrier surrounding the central nervous system (BBB, blood–brain barrier) [7•] and their strong binding to nAChR in the bee's central nervous system are responsible for a unique chronic and sublethal toxicity profile. Neonicotinoid toxicity is reinforced by exposure time. Some studies indicate a non-monotonic [162•] dose–response curve at doses far below the LD50. Mass bee dying events in spring from acute intoxication have occurred in Germany, Italy, Slovenia and France during pneumatic sowing of corn seeds coated with neonicotinoids. Bees that forage near corn fields during sowing get exposed to acute lethal doses when crossing the toxic dust cloud created by the sowing machine.

At field realistic exposure levels, neonicotinoids produce a wide range of adverse sublethal effects in honeybee colonies and bumblebee colonies, affecting colony performance through impairment of foraging success, brood

and larval development, memory and learning, damage to the central nervous system, susceptibility to diseases, hive hygiene etc. Neonicotinoids synergistically reinforce infectious agents such as *N. ceranae* and exhibit synergistic toxicity with other agrochemicals. The large impact of short term field realistic exposure of bumblebee colonies on long term bumblebee queen production (85% reduction) could be a key factor contributing to the global trends of bumblebee decline. Only a few studies assessed the toxicity to other wild pollinators, but the available data suggest that they are likely to exhibit similar toxicity to all wild insect pollinators. The worldwide production of neonicotinoids is still increasing. In view of the vital importance of the service insect pollinators provide to both natural ecosystems and farming, they require a high level of protection. Therefore a transition to pollinator-friendly alternatives to neonicotinoids is urgently needed for the sake of the sustainability of pollinator ecosystem services. The recent decision by the European Commission to temporary ban the use of imidacloprid, thiamethoxam and clothianidin in crops attractive to bees is a first step in that direction [163].

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Environmental relevant concentrations of imidacloprid negatively affects ovary development and fecundity in bumble bees. Dietary imidacloprid at 1 $\mu g\ l^{-1}$ is capable to reduce brood production by one third. The causal speculations include a feeding reduction linked to toxic exposure and/or alteration of the social communication among bumblebee workers.

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This study demonstrates the joint effect of a pathogen microsporidia (*Nosema ceranae*) and imidacloprid on honeybee health. A synergistic effect appears whenever honeybees are exposed to environmental relevant doses of the latter. Significant reduction of the enzyme glucose oxidase, enzyme linked to the social immunity of the colony, was observed in the groups exposed to both stressors.

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