Neonicotinoids, bee disorders and the sustainability of pollinator services

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Abstract
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 in 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, larval development, memory and learning, damage to the central nervous system, susceptibility to diseases, hive hygiene etc. Neonicotinoids exhibit a toxicity than 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.

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 allowed 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 post-synaptic membrane, mimicking the natural neurotransmitter acetylcholine by binding with high affinity [3-8]. 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 binding sites in vertebrate nAChRs in the 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 authorized in more than 120 countries for more than 1,000 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 to 20% of the amount of active substance applied actually enters the crop to protect it [20], and the remaining 80 to 98.4% pollutes the environment without any intended action to plant pests. Diffusion and transformation of pesticides in the environment leads 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]. Due 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 to 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 imidaclolprid, 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-
6,900 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 of its residues in pollen, nectar, sowing dust etc., to non-target pollinating insects became clear. This led to various harmful effects [10, 37-43].

**Ecosystem services of pollinators**

Amongst the wide diversity of pollinating species [44], bees are the most important. Although bee research mostly focusses 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 [45-51]. 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 [72-75]. 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 sensibility 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 analyzed 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 [80-82]. 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 authorized 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 traveling 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: (a) intake of food that contain residues; (b) nesting material (resin, wax etc.); (c) direct contact with spray drift and dust drift during application; (d) contact with contaminated plants, soil, water; (e) use of cooling water in the hive; and (f) 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 sub-divided 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, bee bread, 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]. Based on data from authorization authorities, neonicotinoids residues in nectar and pollen of treated crop plants are estimated to be in the range of below analytical detection limit (0.3 µg kg⁻¹) to 5.4 µg kg⁻¹ in nectar, the highest value corresponding to clothianidin in oilseed rape nectar, and a range of below detection limit (0.3 µg kg⁻¹) to 51 µg kg⁻¹ in pollen, the highest value corresponding to thiamethoxam in alfalfa pollen [85]. A recent review reports wider ranges: for pollen: >0.2 to 912 µg kg⁻¹ for imidacloprid and <1.0 to 115 µg kg⁻¹ for thiacloprid [92]. Residues of imidacloprid, dinotefuran, and thiamethoxam plus metabolites in pumpkin
treated with United States label rates reaches average levels up to 122 µg kg\(^{-1}\) in pollen and 17.6 µg 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 has 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 d 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 µg 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 µg kg\(^{-1}\) (acetamiprid) to 153 µg 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]. *Osmia lignaria* is more sensitive to both clothianidin and imidacloprid than is *B. impatiens*, with *Megachile rotundata* more sensitive still [100]. In an acute toxicity test under semi field condition on the Indian honeybee *Apis cerana indica*, clothianidin showed the highest toxicity, followed by imidaclorpid 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 [104-109]. 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 cease 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 1/10th 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 [117-122]. 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 to 100% of the colonies exposed to imidacloprid contaminated food at 20 µg kg⁻¹ [123] and 80-120 days for 1 mg kg⁻¹ dinotefuran and 400 µg 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 (for example, 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 (for example, each 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 sub-lethal 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 µg l⁻¹) imidacloprid repels pollinating beetles while at concentrations well below the analytical detection limit (0.01 µg 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 µg 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 [127-130] and the orientation and foraging activity [131]. The impact of sublethal exposure on the flying behavior and navigation capacity has been shown through homing flight tests [82,126,132,133]. Exposed to a very low concentration (0.05 µg kg⁻¹) imidacloprid honeybees show an initial slight increase in travel distance. However, with increasing concentration, starting at 0.5 µg kg⁻¹ imidacloprid decreases distance traveled 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 imidacloripid 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 (EC₅₀) of 3.7 µg kg⁻¹ [102]. In queenright bumblebee colonies foraging in a glasshouse where food was 3 m from their nest, 20 µg
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 fulfil 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 [140-142], 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 lead to a delayed development of honeybee larvae, notably in the first state (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 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 authorization of the use of neonicotinoids is essentially based were not developed to detect sublethal nor long-term effects on the colony level, and the observations of the performance 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 is 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 groups, 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 field tests, and subsequently demonstrated that at exposure to field realistic doses, imidacloprid does have significant sublethal effects, even at authorized 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 need not be representative of the range of effects that could occur in real conditions. Due to the large variability of factors that cannot be controlled (e.g. other stressors, soil structure, climate, combination of plants attractive for 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 cause 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/reportPS2371Mar13.pdf]. However, it turned out that the control colonies themselves were contaminated with the pesticides tested. Further, thiamethoxam was detected in two out of the three bee groups tested, even though it was not used in the experiment. [147] 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 how many individuals 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 & 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 (azole s, 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 1,141 depending on the combination \[151,152\]. The strongest synergism has been found for triflumizole making thiacloprid 1,141 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 \[39, 154-156\]. 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 exposure to neonicotinoids that impairs grooming, is well known and often used in pest management of target insects \[158-161\].

**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 use. 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 Nosema 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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References and recommended reading

Papers of particular interest, published within the period of review have been highlighted as:
* of special interest
** of outstanding interest

doi:10.1021/jf103856c


Excellent review of neural impacts of field exposure of honeybees to sublethal residues of neurotoxic insecticides in pollen and nectar. These impair (a) cognitive functions, including learning and memory, habituation, olfaction and gustation, navigation and orientation; (b) behaviour, including foraging and (c) physiological functions, including thermoregulation and muscle activity. Time is a key factor in insecticide toxicity. Combination toxicity of joint exposure to multiple pesticides urgently requires attention.

Introduction into the molecular basis of binding site interaction and explanation of different binding affinity of neonicotinoids with insect and vertebrate nicotinic acetyl choline receptor. Another key factor explaining the high insect toxicity is its capacity (stemming from hydrophobicity) to penetrate the “ion-impermeable barrier” surrounding the insect nervous system.

doi:10.1038/ncomms2648
Using recordings from mushroom body Kenyon cells in acutely isolated honeybee brain, it is shown that the neonicotinoids imidacloprid and clothianidin, and the organophosphate miticide coumaphos oxon, cause a
depolarization-block of neuronal firing and inhibit nicotinic responses. These effects are observed at concentrations that are encountered by foraging honeybees and within the hive, and are additive with combined application. Exposure to multiple pesticides that target cholinergic signalling will cause enhanced toxicity to pollinators.


23. Haith DA: Ecological risk assessment of pesticide runoff from grass surfaces. *Environmental science*


27. Van Dijk T, Van Staalduinen M, Van der Sluijs JP: Macro-invertebrate decline in surface water polluted with imidacloprid PLOS ONE 2013, 8:e62374. doi:10.1371/journal.pone.0062374.


Bees are exposed in many ways to neonicotinoids in agricultural fields throughout the foraging period.
During spring, extremely high levels of clothianidin and thiamethoxam were found in planter exhaust material produced during the planting of treated maize seed. Neonicotinoids were found in soil also of unplanted fields. Dandelions visited by foraging bees growing near these fields contained neonicotinoids: 1.1 to 9.4 µg kg⁻¹ clothianidin and 1.1 to 2.9 µg kg⁻¹ thiamethoxam.


Synergism was found between neurotoxic insecticides and *Nosema ceranae* infections: Sublethal doses of fipronil and thiacloprid makes honeybees more prone to *Nosema ceranae* infections and induces increased mortality of nosema-infected bees. This effects does not seem to be linked to a decrease of the bee detoxification system. A step forward in understanding the interactions of factors jointly producing colony depopulation.


Using the bans of imidacloprid in France for sunflower (1999) and maize (2004) as a case study, the social processes that ultimately lead to application of the precautionary principle are analyzed in a very insightful way. The analysis focuses on the ways in which scientific findings were used by stakeholders and decision-makers to influence policy during the controversy. Eight lessons are drawn about governance of controversies related to chemical risks.


This paper explores the politics of expertise in an ongoing controversy in the United States over the role of neonicotinoids in colony collapse disorder. A set of research norms and practices from agricultural entomology came to dominate the investigation of the links between pesticides and honey bee health. The epistemological dominance of these norms and practices served to marginalize the knowledge claims and policy positions of beekeepers in the colony collapse disorder controversy.
45. Klein AM, Vaissière BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, Tschäntke T:  

The first spatially explicit map of pollination demands at world level identifies the areas that benefit and depend the most on pollination. Based on this maps the dependency of agriculture on pollination is much higher than what has been so far described. Increases in the price of pollination dependent crops were considered as an early warning tool on the abundance of pollinators. Still the effects of international trade of pollination-dependent crops have not been taken into account, in which case the dependency of developed countries would be higher than shown.


Pollinator mediated crops account for >90% of vitamin C, 100% of lycopene, almost 100% of the antioxidants β-cryptoxanthin and β-tocopherol, the majority of the lipid, vitamin A and related carotenoids, calcium and fluoride, and a large portion of folic acid. Ongoing pollinator decline may thus put the provision of a nutritionally adequate diet for the global human population at risk.

70. Burkle LA, Marlin JC, Knight TM: Plant-Pollinator Interactions over 120 Years: Loss of Species, Co-Occurrence, and Function. Science 2013, 339. 1611-1615. DOI: 10.1126/science.1232728
71. UNEP: Global honey bee colony disorders and other threats to insects. Agriculture United Nations Environmental Program; 2010.

This publication gathers the to-date most harmonised information on colony losses worldwide. Colony bee losses are voluntarily communicated by beekeepers through a common worldwide questionnaire designed by the scientific network COLOSS.

73. VanEngelsdorp D, & Meixner M: A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *J Invertebr Pathol* 2010, **103**:S80–95. doi:10.1016/j.jip.2009.06.011


A survey carried out to beekeepers over three consecutive years identified large numbers of bee losses due to acute intoxications to putatively neonicotinoids. The colonies around rice and orange fields were the most affected.


An insightfull discussion of a hypothetical link among biogenic amines-based pesticides (neonicotinoids and formamidines) and their disruptive effects on biogenic amine signaling causing olfactory dysfunction in honeybees. The hypothesis that chronic exposure disrupts neural cholinergic and octopaminergic signaling in honeybees is supported by the fact that abnormality in biogenic amines-mediated neuronal signaling impairs their olfactory learning and memory. This explains why foragers exposed to neonicotinoids fail to return to their hive – a possible cause of CCD.


Colonies of bumble bees (*Bombus terrestris*) exposed to field relevant doses of imidacloprid showed significantly lower growth rate and a reduction of 85% of new queen production. This means a severe negative impact on the bumblebees' population.


Bees exposed to doses of thiamethoxam at 1,34 ng/bee significantly decrease their ability to carry out their homing flight up to levels triggering depopulation dynamics in their colony. RFID tagging technology is used to register the activity of foragers leaving and entering the hive.

**83.** Stoner KA, Eitzer BD: *Movement of soil-applied imidacloprid and thiamethoxam into nectar and pollen of squash (Cucurbita pepo).* *PLOS ONE* 2012, 7:e39114. doi: 10.1371/journal.pone.0039114


This non-comprehensive, but rather complete review, includes information about many of the following subjects: (1) a definition of specific protection goals linked to pollinators; (2) exposure paths of bees to pesticides defined in scientific literature; (3) toxicological effects observed; (4) synergistic effects of pesticides and pathologies and among pesticide molecules, (5) an analyses of the different toxicological tests currently carried out for risk assessment. On the basis of the previous points, a new proposal for pesticide risk assessment on bees is proposed.


Murthy R: **Comparative toxicity of certain insecticides against *apis cerana indica* under semi field and laboratory conditions.** *Pestology* 2011, **35**:23-26.


Thanks to a quick analytical method, new potential sources of exposure to pesticides (clothianidin and thiamethoxam) in the environment have been proved: significant amounts of coating particles are emitted by pneumatic drilling machines during corn sowing. Coarse particles seem to sediment closer to the sown field, while fine particles cover longer distances thanks to the wind. The amount of toxic compound released is toxic to bees, and this toxicity is facilitated by humidity.


110. Gross M: **Pesticides linked to bee deaths.** *Current Biology* 2008, **18**:684.


Neonicotinoids have an irreversible impact on nAChR which implies that a time-to-event model of toxicity is the adequate description of their chronic toxicity profile. Because of time dependence of the toxic effect, the standard risk assessment procedures are not valid in case of exposure to sublethal concentrations of neonicotinoids for long periods of time, so LC50 is a misleading indicator for harm.


A short term sublethal dosage of imidacloprid given to honeybee larvae has a long term effect: it renders the olfactory associative behavior of the adult bees impaired. This may affect the survival condition of the entire colony, even though the larvae survive to adulthood. Also, the brood-capped, pupation, and eclosion rates of the larvae decreases significantly with dose.


Video-tracking technology has been used to measure effects on behaviour of two pesticides: tau-fluvalinate (topical) and imidacloprid (oral). While at very low dose (0.05 µg kg⁻¹) imidacloprid showed an activating effect, starting at 0.5 µg kg⁻¹ imidacloprid triggered a dose dependent negative effect: an increase in time spent at feeder, while distance travelled and interaction time between bees decreases with dose.


Chronic exposure of bumble bees to a neonicotinoid (imidacloprid) and a pyrethroid (λ-cyhalothrin) at field-relevant concentrations reduced the foraging performance and increased worker mortality. This leads to a reduction of brood production and colony success. A synergistic effect of both pesticides is clearly observed.


Environmental relevant concentrations of imidacloprid negatively affects ovary development and fecundity in bumble bees. Dietary imidacloprid at 1 µg 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.


Imidacloprid administered under laboratory conditions to honeybees at 2 µg kg⁻¹ in sugar solution and 3 µg kg⁻¹ in pollen pastry has sublethal effects on the development of the hypopharyngeal glands (HPGs) and respiratory rhythm. The acini, the lobes of the HPGs of imidacloprid-treated honeybees, were 14.5 % smaller in diameter in 9-day-old honeybees and 16.3 % smaller in 14-day-old honeybees than in the same-aged untreated honeybees. Imidacloprid also significantly affected the bursting pattern of abdominal ventilation movements (AVM) by causing a 59.4 % increase in the inter-burst interval and a 56.99 % decrease in the mean duration of AVM bursts.

143. van Tome HV, Martins GF, Lima MAP, Campos LAO, Guedes RNC: Imidacloprid-induced impairment of mushroom bodies and behavior of the native stingless bee melipona quadrijaculata


This meta-analyses shows that dietary imidacloprid at field realistic doses is able to induce sublethal effects at colony level under either acute or chronic regimes (between 6% and 20% performance reduction). The statistical power of the existing field tests that claim to show no effects of neonicotinoids on honeybee colonies is insufficient to support a claim that such effects don’t exist.


149. Moritz RFA, Southwick EE: Bees as superorganisms: an evolutionary reality. Springer-Verlag; 1992


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.


Nosema infection increases significantly whenever bee colonies are exposed chronically to sublethal levels...
of the neonicotinoid, imidacloprid. 5 and 20 µg kg⁻¹ imidacloprid were administered to pollen patties and provided to bee colonies over 5 and 8 weeks.


159. Quintella ED, McCoy CW: Pathogenicity enhancement of Metarhizium anisopliae and Beauveria bassiana to first instars of Diaprepes abbreviatus (Coleoptera: Curculionidae) with sublethal doses of imidacloprid. Environ. Entomol 1997, 26:1173-1182


Researchers say that some chemicals have unexpected and potent effects at very low doses. The complex interplay of receptor binding and gene reprogramming can generate bizarre dose-response relationships, many of which are still being mapped out. Regulators aren’t convinced.