Assessment of pesticides risk for bees: methods for PNEC measurements

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Abstract

Background: An individual honeybee shows a complex behavioral structure. Each bee takes part in the collective behavioral set up that ensures bee colony survival and development. Contaminants are likely to have effects on individual bees' behavior with consequences at the level of the whole colony. They also are likely to alter bees' physiology, including lifespan, fertility or fecundity, leading to colony weakness or colony collapse.

Results: Peer-reviewed scientific literature provides a wide range of methods used for testing honeybees' behavioral or physiological parameters. Apart from alterations that may appear during the conduction of acute or chronic toxicity tests, specific tests could be conducted to complement the risk assessment in order to evaluate the impact of sublethal doses of contaminants on bees. Such tests can be developed both in laboratory conditions or as part of the semi-field and field tests that are currently required as higher tier tests of risk assessment schemes.

Conclusion: The purpose of this work is to review some of these methods and discuss their relevance in the evaluation of pesticide active substances and/or products in view to propose their future inclusion in pesticides risk assessment to bees.

Keywords: honey bee, sublethal effects, risk assessment

1. Introduction

Prior to introducing pesticides on the market, these products must be assessed in compliance with European regulations (Regulation (EC) 1107/2009). This assessment is performed following the annexes of the Regulation and EPPO guidelines (EPPO PP 1/170 (4), EPPO PP 3/9). However, the guidelines and the current assessment scheme currently applied to the risk posed to bees are no longer relevant for assessing systemic pesticides that are likely to be available for bees through water, air (sowing contaminated dust), nectar or pollen (Alix and Vergnet 2007).[1] Systemic pesticides might be used both as soil or seed treatment as well as in spray. Regardless of the way of application, the potential to contaminate pollen or nectar has already been proven (Villa et al.2000, Ham et al. 2006).[2,3]

These substances raise the problem of assessing substances that bees are orally exposed to, through their food sources. Bees are faithful to their food sources (pollen and nectar). Therefore, if these matrices are contaminated, the exposure of bees will lengthen in time. The way the different castes and classes of bees will be exposed differs according to their function within the hive. Foragers may be exposed continuously along the flowering time of the crop/plant, sometimes during their whole forager life. Moreover, in the hive, pollen and honey stocks are likely to be contaminated too. As a consequence, food stocks consumption may lead to a prolonged and continuous contamination for all bee categories in the hive. Regardless the type of bee considered, systemic pesticides induce an exposure to low doses of molecules, often not able to induce acute mortality, but extended in time.

Several scientific studies have proven the impairment of bees' behavioral or physiological abilities after exposure to low doses of pesticides (Desneux et al.2007).[4] The consequences may affect the whole colony, leading to a honey production decrease, colony stress and weakness and potentially to colony death. Khoury *et al*. (2011)[5] show that a decrease in workers lifespan may conduct to the collapse of the colony. Since the exposure to lower doses of pesticides is extended in time, a relevant assessment scheme should include a careful assessment of chronic effects as well as sublethal effects.

The present article focuses on sublethal effects of pesticides on bees' physiology and behavior. It aims to expose the limits of current methods assessing sublethal effects and to make a short review of

various validated methods performed either in laboratory, in field or semi-field conditions in order to measure some bees biological parameters that are - or could be - used for assessing sublethal effects of contaminants present in water, air or food.

2. Current assessment of sublethal effects

Currently, sublethal effects are sometimes assessed in higher tier tests only through field and semi-field tests performed on the whole colony. Such an option is based on the fact that these tests are the most representative of actual field conditions. However, this evaluation structure raises some concerns because tunnel and field trials show several limitations when applied to substances having slow, indirect, chronic and/or delayed effects.

2.1. Limitations of semi-field trials

A first shortcoming concerns bee brood development assessment. Bees are disturbed in tunnels because of confinement. Confinement effects can be observed on foragers (some bees are always seen stuck on the tunnel gauze and seem to be disoriented) but it has effects on hive bees too: bee brood rearing is impaired in tunnels. The brood termination rate in tunnels is usually lower than the one of free foraging colonies (Giffard H, 2011, pers. comm.). After some weeks, brood surfaces decline in the colonies and the lack is total for some of them (see for instance the assessment dossier of the a.i. fipronil).[6] A comparison between bee brood in tested item and control allows some observations but bee brood assessment in semi-field testing has got some inconvenient limits:

- absence of difference between control and test item hives does not allow to conclude that the tested substance has no effects on bee brood success since the confinement effects could mask the substance effect
- observation of delayed or long-term effects is impossible in semi-field trials.

Hence semi-field trials are not suitable for assessing quantitatively the effects on bee brood and do not allow establishing LD50 for larvae.

A second limit is that tunnel methods are not replicable because tested product may be stored into the combs and sometimes diluted in nectar before it is consumed by larvae (Aupinel et al. 2007).[7]

A third limit is that bees' exposure in tunnels is not comparable to actual exposure in fields. Although when a product is sprayed, the way bees are exposed is the same in tunnel as in field, when applied as soil or seed treatment and available through water, nectar or pollen, the bees' exposure in tunnel cannot be considered as representative of such an exposure in field since the flower patches areas are smaller than in field. Moreover, these testing areas are unable to cover the colony needs so that the available flowers are 'over-foraged', leading to two consequences: (1) bees can show abnormal foraging behavior that are likely to mask the effects the substances can have on this behavior, and (2) the amount of nectar or pollen collected by bees cannot be considered as representative of the amount that bees usually harvest in field: bees are under-exposed in tunnels compared to actual field conditions. Moreover no toxic reference can be applied to estimate the bees' exposure.

Thus semi-field tests are not sufficient for assessing effects on bee brood, delayed and long-term effects on the colony, and some behavioral effects that can be masked by confinement effects of the tunnel.

2.2. Limitations of the field trials

Field trials are suitable since they are the most representative of the actual conditions bees will face when the substance or product will be put on the market. However such trials raise some concerns when they are applied to systemic substances used in soil or seed treatment.

The first one is concerning colonies' exposure. Even in large surface tested fields (for instance 3 ha), this surface remains well below the usual bee forage areas when the product is used at the agricultural market level. Unsurprisingly when the treated field is the only resource bees can forage, the level of this harvesting is well below normal harvesting (for instance in an assessment dossier[6] a field study on sunflower shows an increase of hive weight of 3,54 kg in the treated item, and a

decrease of 0,4 kg in the control item during exposure (12 days) when a 'normal' honey harvesting on sunflower is 60 kg on the same period (ACTA1998)[8]). On the contrary, when the harvesting level appears normal despite the restricted area of treated flowers, it usually means that bees foraged in fields outside of the study. In both cases bees are underexposed in comparison to the exposure they will be submitted to, once the product is on the market.

A second issue is the difficulty to find adequate control fields. Maize or sunflower are unable to grow normally in areas that are not allotted to such crops. The trials are thus usually performed in areas where these crops are usually grown. In such areas the pesticide use is a standard practice so that the presence of a contaminant background is practically unavoidable. When the effects that the study aims to check are sub-lethal, chronic or of a delayed-type, it is often problematic to discriminate them from the background ones.

A third limit is that field tests are statistically valid for detecting clear-cut effects only. Performing field trials is expensive (i.e. because fields treated with non-authorized products must be canceled out), therefore only a few field tests are submitted in an authorization dossier. Moreover, the high variability between hives and climatic or weather conditions needs a large sample size for detecting effects at an acceptable level. A statistical analysis of four field trials performed with imidacloprid found that two studies only were statistically able to detect a reduction in bees' performance, the first one if the reduction in honey harvesting is superior to 56% and the second one if it is superior to 33% (Cresswell et al. 2010).[9] The same study states that *the probability that all* [studies] *would fail independently to detect statistically the largest predicted field-realistic sub-lethal effect is (…) 36%, which is unacceptably high for concluding that sub-lethal effects are non-existent*.

Field tests as currently performed are thus insufficient for assessing sub-lethal effects that could lead in the long term to colony weakness and sometimes to colony collapse. For instance in Khoury *et al.* (2011),[5] the colony will collapse when the foragers lifespan is chronically reduced from 6,5 to 2,8 days, i.e. a reduction of 57% of an individual bee lifespan. The ability of current field tests to detect such lifespan reduction is uncertain.

Field trials are the only ones able to assess some parameters (for instance, for assessing quantitatively the queen's egg laying, i.e queen fertility, since the full power of egg laying is reached in large colonies only). Improving their reliability requires setting up of consistent methods to measure bees' exposure and sub-lethal effects of the tested products on bees using enlarged sample sizes in order to obtain statistically validated results. Yet it will still be difficult to ensure a bee's exposure closely representing the reality when the tested products are used in soil or seed treatment.

It appears thus necessary to adopt a more protective approach when assessing the risk of pesticides to bees, especially when sub-lethal effects may be expected. Sub-lethal effects on bees' behavior and physiology should be tested specifically, through laboratory and field studies on individuals or on the whole colony.

3. Place of sub-lethal effects evaluation in assessment scheme

The currently applied assessment scheme is based in the first tier on a hazard quotient i.e. a comparison between the acute LD50 (Lethal Dose 50, a laboratory test on individuals) and the application rate (Hazard Quotient: HQ = application rate (g/ha)/LD50 (µg/bee).

EPPO's last guidance document (Alix and Lewis 2010)[10] proposes the measurement of the acute LD50 and of the contaminant concentration bees are exposed to; at the first assessment tier it proposes to calculate a toxicity-exposure ratio, i.e. a comparison between the predicted environmental concentration (PEC) and the acute LD50 (Toxicity Exposure Ratio: TER = LD50/PEC).

Until this time, the used parameter at first tier is thus a mortality test. Both schemes consider acute toxicity only, an option that is open to criticism when applied to substances that are available in pollen or nectar the whole blossoming period long. Indeed in that case of chronic bees' exposure (continuous exposure) the resulting toxicity cannot be inferred from the acute toxicity measurements: for a lot of substances LD50 by continued exposure during 10 days is different from

LD50 by a single acute exposure, difference factor between them may reach several tens or several hundreds (Decourtye et al. 2005).[11]

Both schemes are thus not protective enough. A more protective scheme should be conceived. It should be based on a first measurement of sub-lethal effects at the individual level in order to discriminate low-risk substances from toxics that are likely to cause impairments to bees' physiology or behavior; so the overall assessment scheme would examine at first tier risks at the individual level, and at higher tier risks at the whole colony level. This option is compliant with SANCO's guidance document 10329[12] since realistic conditions involve colonies rather than individuals.

Some of the methods reviewed in the next part of this article are laboratory tests, some others field tests. Field studies can assess individual behavior alterations (for instance orientation ability) or colony behavior modifications (for instance lifespan: bees' lifetimes in laboratory or in hive are basically different since bees' survival involves relations with the nest mates in the hive as superorganism). For being compliant with the cited scheme rationale, we propose to apply the individual tests at the first assessment tier, the colony level tests at the higher tier.

All these methods could be used or adapted for establishing a PNEC (Predicted Non Observable Effect Concentration) and comparing them with PEC (Predicted Environment Concentration), allowing the establishment of a PEC/PNEC risk coefficient.

4. Existing methods for bees' physiology parameters measurements

4.1. Biomarkers

Like for human being or other vertebrates, there are biological parameters that are able to indicate toxic effects on invertebrate physiology (Hyne and Mayer 2003).[13] Acetylcholinesterase (AChE) can be used as a biomarker of neurotoxicity and exposure to deltamethrin in honeybee (Badiou *et al.* 2008).[14] Fenitrothion and cypermethrin lead to decreases in Na+/K+ ATPase and acetylcholinesterase (AChE) activities in emerging bees (Bendahou et al. 1999).[15] Due to the importance of Na+/K+ ATPase in the energetic metabolism, this can cause dysfunctions at cellular level, i.e. in the cardiac muscle (Desneux et al. 2007).[4] Imidacloprid increases the level of cytochrome oxidase in the mushroom bodies of honey bee brain; this modification is related to the impairment of the medium-term olfactory memory (Decourtye *et al.* 2004).[16]

Exposing bees to a contaminant can modify other enzyme functioning. Enzymes of the oxidant stress (superoxide dismutase, glutathione reductase, glutathione peroxidase and catalase), of the immune system at the individual or at the social level, (phenoloxidase, glucose oxidase), and others enzymes likely to be involved in detoxification mechanisms (glutathion-S-transferase, alkaline phosphatase) were successfully used for establishing toxicity profiles of four pesticides: imidacloprid, thiamethoxam, ethiprole and fipronil (Brunet *et al.* 2011).[17]

Malaspina and Da Silva-Zacarin (2006)[18] provide a mini-review of cell markers that could be useful in monitoring bees exposed to pesticides. The study focuses on stress proteins (heat shock proteins) and their relationship with histological damages in bees' midgut and Malpighian tubules.

Biomarkers analyses are cheap regarding the costs of usual trials and need only a few bees (5-6 bees for analysis of 6 biomarkers). The method needs to be developed but already appears from the first available studies to be really suitable for a first screening of the substance toxicity at the first tier of the assessment scheme as well as for monitoring honeybee status in agricultural areas where bees are chronically exposed to low levels of many environmental contaminants (Malaspina and Da Silva-Zacarin 2008).[18]

4.2. Immunity

Immunity leads us once again to biomarkers studies since they are widely based on phenoloxidase and glucose oxidase level analysis. However, they often also include a haemocyte count. Using these three parameters, Alaux *et al.* (2010)[19] showed a synergic interaction between imidacloprid and Nosema microspores.

4.3. Reproduction, fertility and fecundity

4.3.1. Brood development

Dai *et al*. (2010)[20] have studied the effects of deltamethrin and bifenthrin at sub-lethal dose on fecundity, growth, and development of honeybees. They observed the fate of eggs mapped on a transparency and measured daily fecundity, egg weight, larva weight, hatching rate, capping rate, emergence rate, success rate of development, egg stage, unsealed brood stage, sealed brood stage and immature stage. They found that both pesticides affect these parameters. Particularly the global period before emergence was longer in colonies fed with contaminated syrup than in control. This is of significant importance since post-capping times have an effect on mite population growth (Wilkinson and Smith 2002).[21] Aupinel *et al.* (2005)[22] proposes a standardized method for assessing bee brood development *in vitro*. This method was used for testing the effects of dimethoate and fenoxycarb contaminations; it yielded NOAEC (Non Observable Adverse Effect Concentration) measurements.[11] It is already validated at the French national level and could be shortly validated at the European level. It should be performed at the first tier of the assessment scheme as soon as the tested substance is likely to contaminate pollen, alongside the LD50 measurement on adult bees because the toxicity to larvae can differ widely (being higher or lower) from the toxicity to adults and cannot be drawn from the chemical family or from the mode of action of the concerned pesticide (Alix and Vergnet 2007).[1]

4.3.2. Queen rearing.

Studying the effects of pesticides on queen rearing is of particular relevance since beekeepers often complain of re-queening failures. Such failures are often associated to other problems (among others colony losses) where the influence of pesticides is suspected (personal observation). Contacts with contaminants are actually able to cause queening problems: for instance an increased rejection of grafted larvae is observed when the cups wax is contaminated by coumaphos (Pettis *et al.* 2004).[23]

4.3.3. Drones fertility.

Pesticide effects on drone fertility were never specifically studied from a toxicological point of view at this time to our knowledge; nevertheless, useful methods exist in other studies. Motility of drone spermatozoa and its evolution were measured in a study of intra- and heterospecific insemination (Phiancharoen *et al.* 2004).[24]

4.4. Lifespan

Lifespan can be measured in a laboratory test and results can be submitted to a statistical treatment that evaluates lifespan estimation of bees (Dechaume-Montcharmont *et al.* 2003).[25]

Tagging bees cohorts and controlling their daily survival at the hive entrance can be used to test lifespan in field. Such methods have been developed in studies focusing on biological issues, for instance the dependence of lifespan upon flight performance (Neukirsch 1982)[26] or upon brood rearing (Amdam *et al.* 2009).[27]

It is of first importance to assess the contaminants effects on bees' lifespan since a reduction of this parameter can lead to the collapse of the colony (Khoury *et al.* 2011).[5] The first kind of tests (laboratory measurements) could be used at the first tier and the second ones at higher tiers to confirm lifespan decrease at the colony level if such effects were detected in laboratory tests on individuals.

5. Existing methods for bees' behavior parameters measurements

5.1. PER (Proboscis Extension Reflex) trial

PER trial is a well-known method used in various purposes since the reflex was discovered by Kuwabara in 1957 (for a general discussion, see Giurfa and Malun 2004).[28] It is used by a lot of research laboratories for assessing bee's memory and of its susceptibility to various conditions, for

instance sleep deprivation (Hussaini *et al.* 2009)[29], protease inhibitors (Pham-Delègue *et al.* 2000)[30] or pesticides contamination (Guez *et al.* 2001, Decourtye *et al.* 2005, El Hassani AK *et al.* 2005).[31,11,32]

Effects on the conditioned learning are now proved for several pesticides, among others pyrethinoids (Decourtye *et al.* 2004, Decourtye *et al.* 2005),[33,11] neonicotinoids (Decourtye *et al.* 2003, Decourtye *et al.* 2004, Decourtye *et al.* 2005, Guez *et al.* 2003)[34,33,11,35] and phenylpyrazoles (Decourtye *et al.* 2005, El Hassani A.K. *et al.* 2009)[11,36]. However, the correlation between decrease of the response obtained in laboratory tests and foraging performance at the colony level remains uncertain at this time (Pham-Delègue *et al.* 2002).[37] Nevertheless, since PER is the appetitive reflex of the bee, it clearly plays an important role in bees foraging performance.

This test appears now robust and provides a first approach of bee brain central nervous system integrity. For these reasons it appears to be a really suitable trial at the first tier of the assessment scheme.

5.2. Homing flight trials

Homing flight trials test bees' ability for orientation during inbound flight. This ability is of first importance since bees' disorientation results in their disappearance and further in the whole colony collapse. Indeed, foragers produce a pheromone (ethyloleate) acting as an inhibitory factor delaying the onset of foraging behavior (e.g. nurse bees remain nurses for a longer period before becoming forager bees) (Leoncini et al. 2004).[38] Foragers' disorientation and disappearance results in a lack of this pheromone: hive bees early become foragers and the number of nurses decreases. It results in a reduction of brood area; if the forager disappearance goes on, the hive collapses and dies.

There are a lot of methods for assessing bees' orientation ability. The simplest one was used in France by Cerutti *et al.* (unpublished data; INRA Avignon), in order to evaluate influence of thiametoxam (a neonicotinoid pesticide) on homing flight. Bees were captured at the hive entrance when flying out; they were tagged and put in an incubator where they were fed with contaminated or non-contaminated syrup. They were then released 240 m away from the hive. Homing flight time was measured.

This laboratory/field test is suitable at the first tier because it is simple, cheap and checks various abilities of the bees, including orientation, locomotion and memory.

Many other methods exist, including semi-field tests (Vandame *et al.* 1995).[39] RFID microchips were used for assessing pesticides effects (Decourtye *et al.* 2011).[40] This method allows recording the whole flight pattern. It could be used at higher tier for precising toxicity observed in the first tier trials (for instance an increased duration of the inbound flight).

5.3. Maze tests.

A lot of tests involving the orientation ability exist. Some of them are simple. For instance Medrzycki *et al.* (2003)[41] used a simple box for recording bees' movements on a single comb; bees' behaviors were classified and compared between treated and control samples. Such a trial allows assessing bee locomotion and mobility.

Other maze tests are more complex and allow assessing complex abilities like visual learning and matching-to-sample ability. Han *et al.* (2010)[42] successfully uses a complex T-maze with colored marks for assessing sublethal effects of GMO contaminated pollen on bees: Cry1Ac + CpTI shows a non-significant effect compared with negative and positive (imidacloprid) controls.

Various decision-boxes with marked holes were used in a several studies, for assessing pesticides toxicity (Decourtye *et al.* 2009)[43] or for studying honeybee visual cognition (for a review see Benard *et al.* 2006). [44]

5.4. Thermoregulation.

Honey bees colony's thermoregulation involves different behaviors (among others heat production by shivering, i.e. titanic contraction of bee's flight muscles, and ventilation). It radically differs in winter from spring and summer.

As soon as the colony rears brood, the temperature range must be 33-36°C in the nest. Bees raised below this temperature show decreased performance levels (Tautz et *al.* 2003),[45] and *Ascosphera apis* spores germinate when the nest temperature falls below 32°C for more than two hours (Wilson-Rich *et al.* 2009).[46]

In broodless colonies, bees of the cluster core produce heat for ensuring the cluster survival. The core's temperature increases when the exterior temperature decreases for maintaining bees that form the mantle edge at least at 7°C (Fahrenholz *et al.* 1989);[47] below this temperature bees collapse and fall at the bottom of the hive. Winter cluster thermoregulation is an accurate and complex mechanism, aiming to maintain the minimum temperature that allows bees' survival, avoiding energetic waste that would lead to stocks overconsumption and to precocious aging of the cluster bees. Indeed high energy consumptions cause a decrease in winter bees' lifespan Neukirch 1982),[26] a process that can lead to colony collapse if it continues over long periods.

Thermoregulation accuracy and efficiency are thus necessary for ensuring colony development in spring and cluster survival during winter. It involves various abilities, among others temperature sensitivity and shivering thermogenesis. It is thus of first interest to assess thermoregulation abilities since its impairment is likely to bring about serious colony disturbances including the cluster death in winter.

Thermoregulation assays are achieved with infrared cameras. This tool was used for investigating synergistic action between a pyrethroid (deltamethrin) and azole fungicides (Vandame *et al.* 1998).[48] Such measurements were performed in other studies investigating the cluster regulation mechanisms (Stanbentheiner *et al.* 2003)[49] or brooding mechanisms (Bujok *et al.* 2002).[50]

Thermographic studies of individual bees are a matter for the first assessment tier. Such studies can be performed at the colony level as well; they then should be part of the higher tier assessment and achieved when effects are suspected based on the first tier tests.

5.5. Foraging behavior

Foraging behavior impairment can lead to colony decline (Desneux 2007)[4] since brood rearing is linked to harvesting. As a consequence, beekeepers will face important economic losses.

Foraging behavior is often assessed in semi-field or field tests (higher tier tests). Counting bees on flower patches is not sufficient: for being useful, the assessment needs to be based on an observation framework that lists signs of abnormal behaviors such as, for instance, motionless bees on flowers or abnormal cleaning behaviors (Giffard and Manet 2009).[51]

However, when the product is a systemic pesticide applied in soil or seed treatment, field and semi-field tests do not allow measuring the effect intensity related to particular concentrations. Such measurements can be achieved by using an artificial feeding device (Yang *et al.* 2008, Borlotti *et al.* 2003),[52,53] which also allows establishing repellent concentrations.

5.6. Other topics

A lot of bee behaviors are now well known but were never used in pesticide assessment tests to our knowledge. For instance the communicative behavior pattern of honeybees is highly complex. It involves dances (Dyer 2002)[54] as well as sounds (Kirchner 1993)[55] or vibrations signals (Schneider 2004)[56] and plays a crucial role in the colony dynamic as a super-organism. For instance, colony survival and development implies that bees choose the most profitable nectar source; therefore, the recruitment dance behavior is of major importance (Seeley *et al.* 1991).[57] It also implies that the colony regulates its activity level to the resource abundance, a regulation bees achieve with the shaking signal (Seeley *et al.* 1998).[58]

Fundamental scientific articles provide many methods for investigating such behaviors but methods for assessing them quantitatively or qualitatively often do not exist for the moment.

6. Conclusions

Assessing sublethal effects of plant protection substances and products is a major issue of pesticide regulation. Following (EC)1107/2009 Regulation, a pesticide can be put on the market if *it may be expected that it shall have no unacceptable effects on the environment, having particular regard to (…) its impact to non-target species* (including honey bees). Development of new plant protection substances such as pesticide coated seeds has led to a new exposure mode of honey bees to pesticides: substances are less concentrated on the plants but are present in all plant organs including pollen and nectar or in exudation water droplets in small amounts that can be brought back to the hive and induce sub-lethal intoxication of bees at all stages (from larva to foragers) and castes (worker, drone or queen). Sub-lethal effects on individual bees can lead to unacceptable effects at the colony level, including colony death. For this reason, the assessment at the first tier of acute toxicity only is no longer sufficient for substances that are likely to contaminate pollen, nectar and water consumed by bees and thus to poison them by chronic exposure day after day.

Methods for measuring physiological or behavioral parameters provide thus important tools not only for higher tiers of the assessment scheme, but for the first tier as well, since a protective approach of honey bee toxicology should take sub-lethal effects into account from the beginning of the assessment. We hope this mini-review can help to choose and develop methods assessing representative parameters of the honey bee health status for a better protection of our colonies.

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