A comparison of acute toxicity methodologies for *Bombus* spp.

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Abstract

Acute toxicity testing (lethal dose and lethal concentration for 50% of the population; LD_{50} and LC_{50}) is a required component of the first level of pesticide risk assessment. A review of peer-reviewed and ECOTOX database toxicity values was conducted to assess methodology and toxicity value consistency. Bumble bee LD_{50} and LC_{50} tests varied in five key areas: test subject, active ingredient specifications, test solution specifications, test conditions, test procedure. Only recently has a consistent methodology for bumble bee LD_{50} tests been released, but differs substantially from previous methods. Study methodologies have varied in at least one component and comparison of acute toxicity values can differ substantially between studies. Although a current standard, the appropriateness of the contact LD_{50} method of anaesthetisation and test location should be revisited. This work demonstrates inconsistency in current peer-reviewed analysis of acute toxicity to bumble bees and that current standard methods may not be perfected.

1. Introduction

Pollinators are responsible for $235-577 billion dollars (USD) in pollination services annually (IPBES 2016), a majority of which is accomplished by insects and humans are reliant upon particular nutrients from pollinator dependent crops, such as Vitamin A (Klein et al. 2007; Smith et al. 2015). The honey bee (*Apis mellifera*) is considered to be the most important managed insect for agricultural production and is considered a bioindicator for agricultural pesticides (Porrini et al. 2003). Thus, there is opposition to the importance of wild pollinators in crop pollination services (Kleijn et al. 2015) however, the specificity of greenhouse production (Velthuis & van Doorn 2006), the additional benefits to crops with wild pollinators present (Garibaldi et al. 2016), the realization that globally pollinators differ, and that it is short-sighted to rely on a single species for important

pollinator-dependent crops (Allen-Wardell et al. 1998; IPBES 2016; Winfree et al. 2007) makes it clear that more data should be gathered about wild bees. Wild bees are present on many orchard, berry and cucurbit vegetable crops in North America and Europe, providing added pollination benefit to crop quality and yield (Garibaldi et al. 2013). For example, bumble bees (*Bombus* spp.) are heavily managed for greenhouse production (Velthuis & Van Doorn 2006) and two solitary bees, the Alfalfa Leafcutting Bee (*Megachile rotundata*) and the Blue Orchard Bee (*Osmia lignaria*) are used to supplement pollination of alfalfa and tree fruit crops, respectively (Pindar et al. 2017).

Bumble bees are also important wild pollinators, particularly in North America where intricate plant-pollinator relationships have evolved (Ollerton 2017). The use of bumble bee pollination services within greenhouse tomato production has been calculated as over $14 billion dollars (USD) in 2004 (Velthuis & Van Doorn 2006)), however the exact value of bumble bee pollination services remains unknown (Goulson et al. 2011). Sapir et al. (2017) demonstrated enhanced seed set and cross pollination by honey bees when bumble bees are present. A diverse assemblage of pollinators can support the stabilization and resiliency of ecosystem services (Folke 2006). Stabilization is necessary to buffer temporal and spatial changes in resources and particularly when the reaction of any one species within an ecosystem is unknown (Winfree & Kremen 2009). Evidence from Illinois suggests plant pollinator relationships are currently under stress from reduced redundancy, the resiliency of the system is in question and potential further change may cause irreversible damage (Burkle et al. 2013). In an agricultural setting, pesticides, including insecticides, fungicides and herbicides are one of the most heavily studied bee stressors. The neonicotinoids are a popular class of insecticides and have been heavily studied with a mix of results between species in field or semi-field studies (Rundlöf et al. 2015; Woodcock et al. 2016 Cutler & Scott-Dupree 2014; Whitehorn et al. 2012).

To address potential effects of pesticides on bees, protocols to assess the toxicity of pesticides exist. The focus is mainly on *A. mellifera* as it is typically used as a bioindicator for pollution by

pesticides (Porrini et al. 2003) due to the heavy industrial agriculture use. These protocols give an approximation of potential hazard to *A. mellifera* for both pollinator dependent and independent crops. The Organization for Economic Co-operation and Development (OECD) has published guidelines for honey bee acute contact (OECD 1998a) and oral (OECD 1998b) toxicity and the United States Environmental Protection Agency (US EPA) has released a different acute contact protocol (US EPA 2012). There have been continuous attempts to create an acceptable assessment factor from honey bee data to wild bees (Arena & Sgolastra 2014; Heard et al. 2017; Thompson 2015), but there is still debate. An outcome of this assessment may have been the release of bumble bee OECD protocols for acute contact (OECD 2017a) and oral (OECD 2017b) toxicity that can follow the risk assessment scaffold of honey bees. However, *A. mellifera* is a poor model for bumble bees and other wild bees due to the different level of sociality expressed and the level of human management. *A. mellifera* is advanced eusocial, with whole hives of thousands of bees overwintering and new colonies created by fisson, whereas bumble bees and solitary bees overwinter as individuals and in the case of bumble bees found a colony in the spring or in the case of solitary bees mate and establish nests for the laying of eggs. As *A. mellifera* risk assessment ultimately focuses upon colony level effects, the most sensitive exposure periods for wild bees are overlooked (Stoner 2016). Additionally, like solitary bees that have been commercialized, testing bumble bees is not typically required. For example, Canada only tests honey bees as required component for pesticide registration when exposure is possible (Government of Canada 2005). Nonetheless, acute toxicity tests have been completed for bumble bees, which will be the focus of this paper. The objectives were to determine to consistency between LD_{50} and LC_{50} methods and compare the LD_{50} and LC_{50} results for the same active ingredient across studies for bumble bees. We tested the hypothesis that if studies are consistent in the assessment of the LD_{50} or LC_{50} then similar LD_{50} or LC_{50} values for the same active ingredient will be obtained.

2. Methods

A literature search was conducted between March and April 2017 for peer-reviewed articles that assessed the fifty-percent lethal dose (LD_{50}) for bumble bees. The University of Guelph literature database was used, which includes databases such as Web of Science and CAB Direct. Record date limits were set from January 1900 to January 2017. Search terms included were "lethal dose", "toxicity", "acute oral", "acute contact", "method", "methodology" and "*Bombus*". These were combined to search for bumble bee specific toxicity values and methodologies. Papers were cross-referenced with those in the US EPA's ECOTOX database (US EPA 2017). Papers that appeared within the ECOTOX database and the literature search were included in analysis, a total of 38 papers. The rationale for the inclusion of papers represented within both searches was to include values that are used in risk assessment by the US EPA and to ensure the values being compared were from peer-reviewed literature.

The methodology was analyzed for similarities and variances within 5 categories; test subject, active ingredient specifications, test solution specifications, test conditions, test procedure (Table 1). As the solution fed to or applied on a bumble bee can be quite different, an additional category relevant to oral or contact toxicity was also included depending on the type of study performed (Table 2). In order to compare a standardized value, only those papers that assessed the LD_{50} or the LC_{50} are reported here, a total of 6 papers. Toxicity values of the same active ingredient within these papers were recorded for comparison across studies.

Protocols for the assessment of the acute contact and oral LD_{50} for honey bees were obtained from the OECD (1998a; 1998b) and the US EPA (2012) in April 2017. Having been published after the initial search for papers, the protocol for the assessment of the acute contact and oral LD_{50} for bumble bees were obtained from the OECD (2017a; 2017b) in January 2018 and included in the methodologies comparison.

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3. Results and Discussion

3.1. Test Subject

In most cases, the average weights of the bees tested are not reported which may have an impact on the toxicity as noted by Drescher and Guesen-Pfister (1991), van der Steen (1994) and Thompson (2015). When comparing the methomyl oral LD_{50} of workers above and below 220 mg, Drescher and Geusen-Pfister (1991) found quite a difference at the 24-hr assessment (3.02 µg/bee vs 3.808 µg/bee), but reported the same values at the 48 and 72-hr assessments. van der Steen (1994) found a substantial link between the size and the LD_{50} of bumble bees. Comparing the smallest and largest groups based upon the average weight shows the small group of 0.162g produced an LD_{50} of 5.5 µg dimethoate/bee whereas the large group of 0.297g produced an LD_{50} of 13.0 µg dimethoate/bee (van der Steen 1994). The impact of size on relative toxicity has been used to explain why honey bees may be more susceptible to pesticides, but serves more as a generality not true in all cases. For example *Megachilie rotundata* and *Nomi melanderi* react similarly to pesticides, but differ by 3-6 mm in size and both react differently than *Apis mellifera* which is approximately the size of *N. melanderi* (Devillers et al. 2003). Evidence from Cresswell et al. (2012) suggests bumble bees are more sensitive to dietary imidacloprid than honey bees, suggesting that bumble bees many have a lower threshold for neonicotinoids. Based upon the limited data for bumble bees, it is recommended that further assessment occur on different *Bombus* spp. to determine if size within species and between species impacts susceptibility to pesticides.

Size concerns also factor into the volume of test solution fed to the bee. With variation in size there is an argument that there should be variation in the volume given, however how much variation within size is acceptable for the determination of a changing volume within bumble bees has not been addressed in the current literature. Nonetheless, no study has altered the volume for the

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size of the bee, instead attempting to remove average size and age workers from colonies prior to the experiment and administering a fixed volume of test solution.

The current database of LD_{50} values is inundated with *Bombus terrestris*, in fact the only other species to have a reported oral LD_{50} for some active ingredients is *B. lapidarius* and the only other species with a reported contact LD_{50} is *B. terricola*. Only *B. terricola* is native to North America, the other two being part of the European bumble bee fauna. Given that *B. impatiens* is a commercial pollinator in Canada and exposure to pesticides is highly likely, the lack of any LD_{50} for this species prevents a risk assessment from taking place that requires such a value. There have been attempts at the creation of a lethal concentration (LC_{50}) value, (Gradish et al. 2012; Scott-Dupree et al. 2009), however they are overwhelmingly contact toxicity with only one oral LC_{50} value for a commercial formulation of deltamethrin (33.8ppm, Decis 5EC, BayerCropScience; Gradish et al. 2012). Furthermore, the LC_{50} is not measured in the same manner and cannot be used with the current honey bee (EPPO 2010) or general bee (Sanchez-Bayo & Goka 2014) risk assessment calculations. This is not an ideal situation given the importance of this species within greenhouses across the country and the various pesticides they may be exposed to. Better toxicity testing for greenhouse specific chemicals is required as the model *A. mellifera* is not appropriate given that they are not used within greenhouses.

3.2. Active Ingredient Specifications

An active ingredient as defined by the Pest Control Products Act (PCPA) as "a component of a pest control product to which the intended effects of the product are attributed and includes a synergist but does not include a solvent, diluent, emulsifier or other component that is not primarily responsible for those effects" (PCPA 2002). Thus, an active ingredient is a technical or analytical compound typically with >90% purity. The supplier should be acknowledged and the active ingredient tested should be as close to 100% as possible to align with the Pest Control Products Act (2002). However, the OECD considers a commercial formulation to be an active ingredient as well

(OECD 1998a; 1998b; 2017a; 2017b). A commercial formulation can include other components and therefore is not an active ingredient according to the PCPA. This distinction is important as there is variation in the amount of the active ingredient within products. For example, sulfoxaflor can be 50% in a water dispersible granule product (Transform WG Insecticide, Dow AgroSciences) or 24% in in a suspension concentrate (Closer Insecticide, Dow AgroSciences). The amount of active ingredient put into the environment can also be adjusted based upon application instructions, Integrated Pest Management (IPM) decisions and farmer error.

The variation in amount of active ingredient being tested for LD_{50} purposes is a cause of concern as there can be differential toxicities assessed between the technical and the commercial formulation. For example, sulfoxaflor and flupyradifurone have different acute oral lethal dose (LD_{50}) values for honey bees between the technical and commercial formulations (Figure 1). In both cases the active ingredient and commercial formulation do not have the same value. Thus, a base line toxicity test may be conducted with the technical compound, but should always be followed up with the specific formulations that will be applied in the environment.

3.3. Maintenance Solution Specifications

The OECD (1998b) guidelines for oral toxicity stipulate a 50% w/v sucrose solution, however some studies utilized honey. The potential impact of honey as compared to sucrose with an active ingredient is not known. However, honey does have a greater tendency to ferment, requiring more frequent replacement of the feeder solution, but bees are able to locate honey faster due to the stronger odour (Pomeroy & Plowright 1980). More research is required to determine if it is best for sucrose or honey to be used in test and control solutions.

3.4. Test Conditions

The laboratory conditions that the bees are kept in may induce a small amount of error across studies as all variables are not held constant. Temperature variations that are not ideal for bumble bees may produce some negative impacts upon worker bees, particularly when they are held

185 individually for days. The OECD (1998a; 1998b; 2017a; 2017b) requirements for honey bees and
186 bumble bees is 25 ± 2°C, but Pomeroy and Plowright (1980) found that natural bumble bee colonies
187 maintained at 29-32°C, with an optimal temperature of 30°C to be the best for long periods.
188 Bees are activated to fly with light, and when confined for the test duration, may become
189 increasingly exhausted or stressed compared to those bees held in constant darkness. This may
190 impact the survivorship of the bees between studies and thus not be totally representative of the
191 toxicity of the active ingredient. However, there has not been research into stress on bees trapped in
192 light and/or dark conditions. It is hypothesized that the most movement would occur at the switch
193 points between light and dark.
194 An underrated aspect of bumble bee survival is the relative humidity of the room in which
195 they are kept. A higher relative humidity allows the supplemental nectar solution to remain moist,
196 rather than hardening as the water evaporates. Previous work conducted in lab suggested that a
197 lower relative humidity was causing solution supplies to run out prematurely and potentially
198 contributing to death rates (personal observation). It would be interesting to determine what relative
199 humidity is maintained within natural colonies in order to mimic these conditions as Pomeroy and
200 Plowright (1980) did with temperature.
201 3.5. Test Procedure
202 Mortality times reported are 24, 48 and 72 hours. In some instances, studies only report a
203 single time point, which can prevents a comparison to other active ingredients if they do not include
204 that same time point. The standard acute toxicity time is 48 hours, but Marletto et al (2003) failed to
205 include this in a large test of oral LD_{50} analysis of *B. terrestris*, providing the 24 and 72 hour
206 results.
207 In some instances, particularly for contact toxicity tests, bees are to be anesthetized. Most
208 often researchers use CO_2 and this is recommended in both the OECD and EPA (Table 3) contact

toxicity guidelines. However, Marletto et al (2003) used a combination of cold and CO_2 to anesthetise the bumble bees, using those that fell under after a few minutes for the toxicity trials (Table 3). Unfortunately, exposure to CO_2 for as little as one second can produce a retarded ability to begin activity over time, even though they are initially more active (Poissonnier et al. 2015). Exposure to cold (5°C) for 13 to 15 minutes initially produces no difference in activity from controls, but overtime demonstrate a greater activity decline than control or CO_2 exposed (Poissonnier et al. 2015). At 96-hr, which would be the final time point in an extended toxicity test, the CO_2 and cold treated bees had failed to regain initial activity levels (Poissonnier et al. 2015), suggesting that the methodology itself interferes with a bees ability to cope with a pesticide. In LD_{50} testing, recording whether or not bees are affected by the test active ingredient is required. If the anesthetic induced behaviour is mistakenly attributed to the test active ingredient then the toxicity profile will be incorrect. Thus, the anesthetic used may be a huge confound in contact testing.

Although not tested for mortality risk, the removal of bees from a nest box with an aspirator and then blowing them out of the tube can cause injury to the bee (personal observation). Depending on the experience of the individual bees can be killed if ejected too quickly from the tube onto a hard surface. To avoid this risk, removal of bees with forceps allows for more control for the placement of bees. It is quite easy to collect bees in this manner when the colony is back light with red light allowing the legs to be visible.

Active ingredients can be dissolved in a variety of mediums, but oral field exposure would only be in nectar, pollen and water. Studies examined adhered to this principle and as discussed previously, either mixed it with a sugar based or honey based solution. For contact exposure, a bumble bee would be sprayed with a tank mixture or land on wetted or dried plant parts. There are protocols for mixtures of specific products, but in a lab test all of these factors may not be replicable. Nonetheless, the application of an active ingredient in acetone is unlikely and leads to an unnecessary control group for acetone application alone in addition to a zero control. Ideally, the

active ingredient should be mixed in water and if examination of the product formulation is to be conducted, the mixture should as closely as possible reflect mixing directions as if it will be applied in field.

3.6. Contact Toxicity

Application amounts vary in contact toxicity tests (Table 3), with honey bee requirements set fairly low by the OECD at 1 µL and bumble bees at 2 µL (1998a; 2017a). Previous work on LD_{50}s ranged from 1 to 10 µL applications and LC_{50}s had 1 to 5 mL applications. With more active ingredient applied, it is hypothesized that the longevity of the bee would decrease thus suggesting a different LD_{50} or LC_{50} value.

The control provided by different application tools may have an impact on toxicity and this is closely tied to the location of the test solution on a bee. A complete spraying of the bee is not ideal and neither is a direct drop where a bee may have access to it. All LD_{50} protocols call for the application to occur on the dorsal thorax (Table 3). Two LC_{50} studies used a potter spray tower for application, which has received criticism due to departure from the standard honey bee protocol of drop application (Table 3; Arena & Sgolastra 2014). However, work completed by Tong and Huang (2018) on grooming intensity and safe pollen sites demonstrated that bumble bees (*B. friseanus* in this study) are capable of intense grooming on the dorsal thorax. The two areas delineated for the dorsal thorax had the most intense grooming of all areas examined on the bee. The application of an active ingredient to a location that is easily and well-groomed by a bumble bee may lead to ingestion of the test active ingredient, therefore confounding the result of the acute contact test with oral exposure.

Assuming the risk of oral exposure should be minimized for any contact exposure test, it would be ideal to spray on sites where the least grooming efficiency is found. The areas along the midline abdomen of the bumble bee, dorsal or ventral were the least efficiency groomed and would

pose the least risk to oral exposure (Tong and Huang 2018). There is no LD_{50} study that compares the same active ingredient with different application locations, but an approximation may be made from two LC_{50} studies. Deltamethrin has been tested with application to the dorsal thorax (Gradish et al. 2012) and ventral thorax (Scott-Dupree et al. 2009) and deltamethrin was found to be 5 times more toxic when applied ventrally (69ppm vs 346.5ppm). However, Gradish et al. (2012), applied only 1 mL and did not report an anesthetic whereas Scott-Dupree et al. (2009) applied 5 mL and used CO_2. Both of these factors may also influence the toxicity.

3.7. Oral Toxicity

Similar to contact toxicity, variations in the amount of test solution given may alter the longevity of bees and affect the calculated LD_{50} or LC_{50}. The amount of test solution fed to the bees has largely not been reported and those reported vary between 10 and 30 µL per bee, which means that bees receive 0.1 and 0.3 µg due to volume change (Table 4). The OECD (2017b) protocols call for 40µL of test solution to be administered to bumble bees, which is larger than anything used previously and there is no justification for this change in the document.

Although the starvation period is longer than honey bees, there is general agreement between all documents, typically ranging from 2-4hours (Table 4). However, the time period allowed to feed is quite different between the OECD protocols for honey bees (1998b) and bumble bees (2017b). The maximum time allowed to consume the solution in studies was a maximum of 2 hours, but at least one LD_{50} and one LC_{50} allowed for 15 minutes (Table 4). Starvation period along with many other changes in methods may have implications on mortality at the times assessed, particularly if the first mortality scoring will be done within 8 hours (Table 5).

A major difference between the oral toxicity tests of bumble bee and honey bees, is the treatment of honey bees as a group of 10 and bumble bees as individuals, which suggests that there may be some issues with direct comparison between the two LD_{50} values. More research into

mortality in light and dark conditions, as well as if bumble bees can be kept in groups to more closely mimic the honey bee protocol is necessary. One argument for the change in methods from the honey bees is that bumble bees do not practice trophallaxis (van der Steen 2001) and bumble bees when kept in microcolonies will display aggressive behaviour until one becomes the dominant (Besard et al. 2010). Nonetheless, it would be interesting to see if these adaptations produced substantially different acute oral LD_{50} values.

3.8. Acute toxicity comparability

Ideally, honey bees would be an appropriate indicator species for wild bees. Honey bees are plentiful in the environment, particularly when they are brought in for pollination services, but are not the most sensitive to pesticide effects (Devillers et al. 2003). When a pesticide reports as non toxic to a honey bee, this does not mean it will respond the same within other species of wild bees, including bumble bees (Devillers et al. 2003). There have been attempts at comparing the LD_{50} of bumble bees and honey bees to estimate which species is on average most sensitive (see Arena and Sgolastra 2014), however the variable methods used to produce these values may influence this comparison. Even with multiple attempts to create an appropriate safety factor (Thompson 2015; Heard et al. 2017), the inability to find one that encapsulates variation suggests that multiple indicator species are required. These indicator species would be spread out across genus to include variation in exposure profiles and sensitive periods. For example, a mated bumble bee queen hibernates over winter and upon emergence, must provision and establish a colony. For about a month (Thompson & Hunt 1999) she is the sole provider for her brood until these workers emerge and can assist in brood care and foraging. With an emphasis on the colony, as in honey bee protocols, the actual sensitive time points will be missed (Stoner 2016). Further, bumble bees also forage at cooler temperatures and at earlier and later times of the day than honey bees (Corbet et al. 1993; Thompson & Hunt 1999). This disparity in flight periods means that exposure to bumble bees can vary quite greatly from honey bees. For example, current EPPO high risk active ingredients are to place warnings on these

products, one of which includes spraying earlier, which would disproportionately impact bumble bees over honey bees. However, the warning of not spraying during flowering can be beneficial to both species. Thus, it is important to consider in what ways current methods of 'pollinator' protection are specifically aimed at *Apis mellifera*.

4. Conclusion

LD_{50} values are calculated to estimate the environmental risk faced when pollinating crops. These risk results then influence how the product is used. For bumble bees, the study methods varied on a least one component of assessment and the comparison of values for the same active ingredient do suggest methods can influence the results. We could not support the hypothesis that consistent methods would produce consistent LD_{50} and LC_{50} results. The differences in methods may or may not impact the results obtained from the analysis. However, based upon study comparison of oral acute toxicity values, there certainly is justification for unease. The most concerning issue, which can vary globally is the definition of an active ingredient, this should be standardized across all laws, policies and protocols. This will ensure similar purity products are being used as pure product and commercial formulations can impact toxicity, such as in sulfoxaflor and flupyradifurone. The lack of consistency in reporting times between studies, can prevent comparison of active ingredients and species.

Bumble bees only recently obtained a standard acute toxicity testing protocol (OECD 2017a; 2017b), which differs from all previous bumble bee acute toxicity assessments. The acute contact toxicity methodology (OECD 2017a) contains some confounding issues regarding the allowance of anaesthetization of bees (Poissonnier et al. 2015) and the placement of the compound in a readily groomed area (Tong & Huang 2018). The acute oral toxicity method (OECD 2017b) has increased the volume of test solution for consumption and extended the acceptable consumption period, the impacts of these changes are not known. More analysis of these factors upon bee behaviour and mortality should be gathered to attempt to predict possible interactions between the

methodology and active ingredient toxicity. Further, as more bee species are used for crop pollination, more protocols should be developed to adequately assess acute toxicity. These new protocols should also consider the differences the exposure profile and sensitive periods, especially as higher level risk assessments become necessary.

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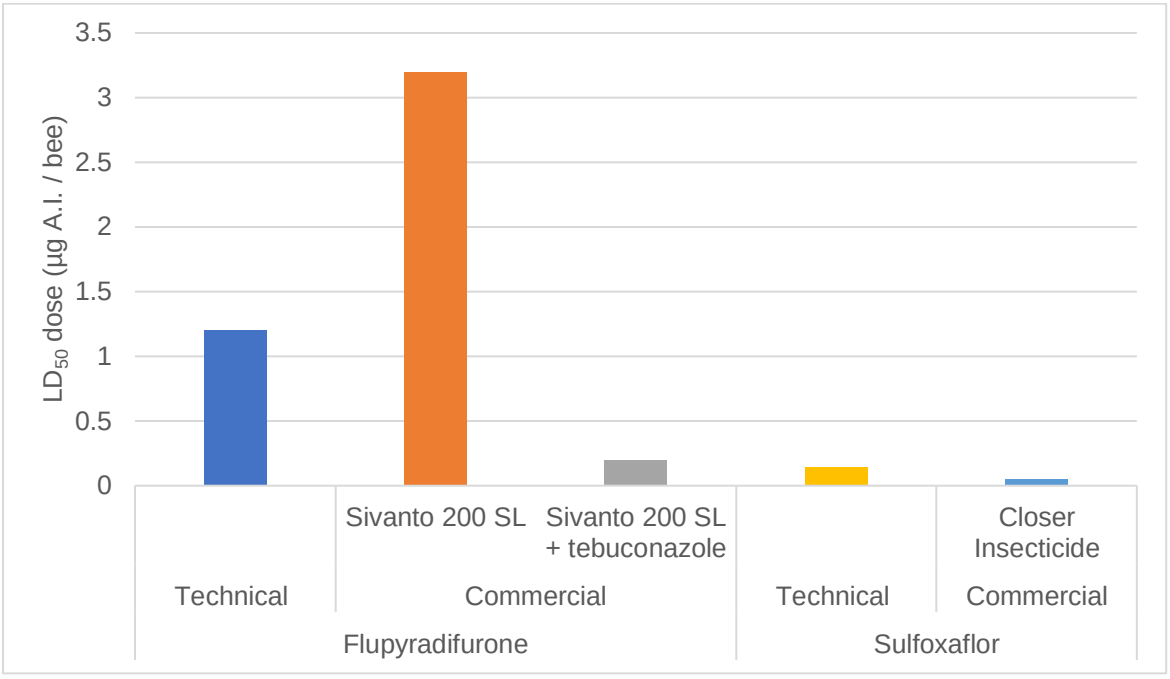

Figure 1. Variation in acute oral toxicity reported as LD_{50} at 48-hrs for the honey bee *Apis mellifera* (Health Canada, 2014; Health Canada, 2015)

Table 1. Methodology information recorded regardless of test type.

Test Subject	**Active Ingredient Specifications**	**Maintenance Solution Specifications**	**Test Condition**	**Test Procedure**
Species	Technical or Commercial	Sugar or honey to sweeten	Light: Dark	Mortality time reported
	Purity (%)	Concentration of sweetener	Temperature	Anaesthetised
	Supplier or Manufacturer		Humidity	Duration of Anaesthetisation
				Removal from colony
				Active ingredient dissolved in

Table 2. Methodology information specific to acute oral or contact toxicity testing.

Oral Toxicity	**Contact Toxicity**
Amount fed	Amount applied
Starvation period	Application tool
Feed allowance	Application location

Table 3. Comparison of methodologies used to produce contact LD_{50} values for *Apis mellifera* (OECD #214 & EPA Guide) and bumble bees as well as the methodology for LC_{50} values for bumble bees (*Bombus terrestris*).

	LD_{50}						**LC_{50}**		
	OECD Honey bee (1998a)	**OECD Bumble bee (2017a)**	**United States Environmental Protection Agency (2012)**	**van der Steen (1994)**	**Helson et al. (1994)**	**Marletto et al. (1993)**	**Scott-Dupree et al. (2009)***	**Gradish et al. (2012)***	**Marletto et al. (2003)**
Applicator	Micro-applicator	Micro-applicator or pipette	Micro-applicator OR Impregnated Dust Chamber		Microdoser/ Microinjector	Automatic Pipette	Potter Spray Tower	Potter Spray Tower	Automatic Pipette
Application Location	Dorsal Thorax	Dorsal Thorax	Dorsal Thorax OR Whole Body	Ventral Thorax Between base of 2nd and 3rd Legs	Dorsal Thorax	Between coxae	Ventral Thorax	Dorsal Thorax	Cage Bottom
Anaesthetic	Carbon dioxide or Nitrogen	Carbon dioxide or Cold	Cold, Carbon dioxide, Nitrogen	-	Carbon dioxide	Carbon dioxide	Carbon dioxide	-	-
Length of Anaesthetic	Exposure should be minimized	-	Minimum amount	-	Temporarily	-	~3 seconds	~10-12 seconds	-
Volume of Solution	1 µL	2 µL	Do not exceed 5 µL	1 µL	2-5 µL	10 µL	5 mL	1 mL	1.1 mL
Light Conditions	Dark	Dark	Dark	-	18L: 8D	Dark	Dark	Dark	Dark
Temperature (°C)	25 ± 2	25 ± 2	25-35	-	16*	28	25 ± 1	25 ± 1	28
Relative Humidity (%)	50-70	40 - 80	50-80	-	60-70	60	-	40-60	60
Expression	µg A.I. bee^{-1}	µg A.I. bee^{-1}	µg A.I. bee^{-1}	µg A.I. bee^{-1}	µg A.I. bee^{-1}	µg A.I. bee^{-1}	% w/v $x10^{-3}$	mg A.I. L^{-1}	ppm

*Bioassay conducted on *B. impatiens*

Table 4. Comparison of methodologies used to produce oral LD_{50} values for the honey bee, *Apis mellifera* (OECD #213) and bumble bee, *Bombus terrestris* as well as the methodology used for LC_{50} values for bumble bees.

	LD_{50}						**LC_{50}**	
	OECD Honey bee (1998b)	**OECD Bumble bee (2017)**	**Drescher and Geusen-Pfister (1991)**	**Marletto et al. (1993)**	**Gretenkord and Drescher (1993)**	**van der Steen (1996)**	**Gradish et al. (2012)***	**Besard et al. (2010)****
Number Treated	Group of 10	Individual	Individual	Individual	Individual	Individual	Individual	Group of 5
Sucrose Solution	50% w/v	50% w/v	50%	50% (Honey)	50%	50%	(Honey)	-
Starvation Period	Up to 2-hr	2-4-hr	-	3-hr	-	2-3-hr	3-hr	-
Solution per Bee	10-20 µL	40 µL	-	10 µL	30 µL	10 µL	25 µL	500mL***
Time Allowed to Feed	3-4-hr	4 hr	-	15 min	-	2-hr	15 min	-
Light Conditions	Dark	Dark	-	Dark	-	Dark	Dark	Dark
Temperature (°C)	25 ± 2	25 ± 2	-	28	25	25 ± 2	25 ± 1	28-30
Relative Humidity (%)	50-70	40-80	-	60	50	-	30	60-65
Expression	µg A.I. bee^{-1}	µg A.I. bee^{-1}	ng A.I. bee^{-1}	µg A.I. bee^{-1}	µg A.I. bee^{-1}	µg A.I. bee^{-1}	A.I. mg L^{-1}	A.I. mg $(L\ fd)^{-1}$

*Bioassay conducted on *B. impatiens*

**Included in the table, but major methodology differences aside from those within the chart

*** This was given to a microcolony so the exact amount consumed by each individual is unknown

518 Table 5. Comparing the LD_{50} acute oral values (µg A.I. bee^{-1}) for *Bombus terrestris* of differing methodologies.

	Gretenkord and Drescher (1993)	van der Steen (1996)	Marletto et al. (1993)		Drescher and Geusen-Pfister (1991)		
Time Point (hr)	**24**	**-**	**24**	**72**	**24**	**48**	**72**
Acephate	-	-	8.36	7.37	135.47	13.40	3.98
Deltamethrin	0.6	0.54	-	-			
Dimethoate	-	1.7	0.44	0.33	-*	-*	-*
Lambda-cyhalothrin	-	0.16	0.21	0.16	-	-	-
Methomyl	-	-	3.46	3.3	3.164, 2.780**	2.752, 2.365**	2.608, 2.177**
Oxydemeton-methyl	0.75	0.76	-	-	-	-	-
Phosalone	60	59.7	3.98	3.98			

519 *Tested but a value could not be determined
520 **Assay was completed with *Bombus lapidaries*