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Concentrations of neonicotinoid insecticides in honey, pollen and honey bees (*Apis mellifera* L.) in central Saskatchewan, Canada

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HIGHLIGHTS

- Pollen, honey and worker bees were extracted by modified QuEChERS method for neonicotinoid insecticides and metabolites.
- Samples collected within a 30 km of the city of Saskatoon indicate the variability inherent within even a short distance.
- Concentration of clothianidin and thiamethoxam based on published LD_{50} values, exceeded the lowest values in 2 Bee samples.
- Imidacloprid metabolite concentrations were at greater concentration than their parent molecule.
- Over winter concentration estimate that dietary intake may impact bee health.

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ABSTRACT

Neonicotinoid insecticides (NIs) and their transformation products were detected in honey, pollen and honey bees, (*Apis mellifera*) from hives located within 30 km of the City of Saskatoon, Saskatchewan, Canada. Clothianidin and thiamethoxam were the most frequently detected NIs, found in 68 and 75% of honey samples at mean concentrations of 8.2 and 17.2 ng g^{-1} wet mass, (wm), respectively. Clothianidin was also found in >50% of samples of bees and pollen. Concentrations of clothianidin in bees exceed the LD_{50} in 2 of 28 samples, while for other NIs concentrations were typically 10–100-fold less than the oral LD_{50}. Imidaclorpid was detected in ~30% of samples of honey, but only 5% of pollen and concentrations were <LOD in bees. Transformation products of Imidaclorpid, imidaclorpid-Olefin and imidacloprid-5-Hydroxy were detected with greater frequency and at greater mean concentrations indicating a need for more focus on potential effects of these transformation products than the untransformed, active ingredient NIs. Results of an assessment of the potential dietary uptake of NIs from honey and pollen by bees over winter, during which worker bees live longer than in summer, suggested that, in some hives, consumption of honey and pollen during over-wintering might have adverse effects on bees.

1. Introduction

Bees and other pollinators responsible for pollination of crops have been an integral part of agriculture for many centuries. Approximately 35% of crops depend directly on pollinators (Klein et al., 2007), accounting for an estimated, annual value of 153 billion Euros (Gallai et al., 2009). The European honey bee *(Apis mellifera)* is the most widely managed pollinator of crops.

Over the past few years, there have been increasing reports of overwintering losses of colonies of bees and significant challenges

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in maintaining healthy colonies in Canada, the US and globally. Maintaining healthy colonies of bees is a complex issue and is affected by multiple factors. According to the Canadian Association of Professional Apiculturists, (CAPA), since 2006, failure of overwintering colonies of bees has increased in Canada and the United States (CAPA, 2013). Failure of overwintering colonies or "colony loss", which is also called "colony collapse disorder" are both terms used to describe colonies that did not survive the winter, which includes colonies that are too weak to survive or died during the early spring. In Canada, national losses of overwintering colonies increased from a historical average of 10–15% to 35% in 2007/08. This was followed by somewhat lesser losses of overwintering colonies from 2009/10 to 2013/14 which ranged from 15 to 29%, (Fairbrother et al., 2014).

While there are many factors that can potentially affect survival of bees, including changes in climate, genetics, changes in nutrition due to changes in cropping patterns from year to year, parasites and viral diseases (Fairbrother et al., 2014), results of some studies have suggested that extensive use of insecticides might be a factor in the increased rates of loss of colonies during the dormant period of winter (Cutler et al., 2014a,b, 2007; Al Naggar et al., 2015a,b). Some bees might be particularly susceptible to pesticides because the European honey bee is deficient in some genes encoding for detoxification enzymes (Claudianos et al., 2006).

Neonicotinoids are one of the most widely used classes of pesticides. In 2010 approximately 20,000 tonnes of active ingredient were used globally which constituted approximately one third of all insecticide treatments (Bonmatin et al., 2015). Their use stems from the fact that they exhibit greater toxic potencies to invertebrates, particularly arthropods compared to vertebrates. They are water soluble but exhibit relatively lesser acute potencies toward fish. Neonicotinoids are persistent in the environment so require less additional spraying, and are versatile in their modes of application (Bonmatin et al., 2015). Currently, neonicotinoid insecticides (NIs) are approved for use as seed treatments, soil applications, and foliar sprays on a wide variety of agricultural crops such as oilseeds, grains, pulse crops, fruits, vegetables, and ornamental plants. Neonicotinoid insecticides are also used on turf, as systemic insecticides injected into trees, in structures and outdoor residential areas, and in pet care products. Seeds of several major crops grown on the Canadian prairies, including canola, wheat, barley, oats and field peas are commonly coated with one of the neonicotinoid active ingredients clothianidin, imidacloprid, or thiamethoxam while acetamiprid is also used on fruit or leafy vegetable crops (Main et al., 2014). Neonicotinoid insecticides are systemic and are accumulated into the tissues of plants, including pollen.

Toxic potencies of NIs to bees vary widely, with LD_{50} values ranging from 2.7 ng bee^{-1} for oral exposure to clothianidin (Laurino et al., 2013) to 14,530 ng bee^{-1} for acetamiprid (EU Commission, 2004). In a study using concentrations as little as 1000 ng mL^{-1} clothianidin and 400 ng mL^{-1} dinitrofuran administered as an oral dose to multiple hives each colony exhibited colony collapse disorders (CCD) or pre-CCD behavior (Yamada et al., 2012). Metabolites or transformation products of some NIs exhibit differing LD_{50} values. For example imidaclorpid-Olefin has a greater toxic potency than that of the untransformed, active ingredient compound (28 ng bee^{-1} compared to 57 ng bee^{-1}), while other transformation products of imidaclopid are orders of magnitude less potent (>1000 ng bee^{-1}; Kamel, 2010). More than 100 transformation products have been listed for NIs with for example clothianidin having some 24 metabolites (Simon-Delso et al., 2015).

Since 2006, NIs have been under increasing scrutiny, but there were no reports of significant mortalities of bees or effects associated with use of NIs in Canada until the spring of 2012, when incidents of mortalities of bees were first reported in some regions of Canada. In 2012, 2013 and 2014, reported incidents related to planting of treated corn and soybean seed were limited to intense corn-growing regions of southern Ontario, with fewer incidents in regions of Quebec and Manitoba where corn is grown. These mortalities were linked to formation of dust containing the NIs by use of pneumatic seed drills used for planting (Alix et al., 2009). Since the method of planting crops on the prairies is different and does not produce as much dust it was uncertain whether similar effects would have been experienced on the western areas of Canada. Over the winter of 2012–2013 the average loss of honey bee colonies in Saskatchewan was 27% (CAPA, 2013).

The 128,000 hives in Saskatchewan are vital to the economy of the province because they provide significant revenue for beekeepers through production of honey and benefits of pollination for many crops. In Saskatchewan, approximately 11.3×10^6 kg of honey is produced annually and 23% of the honey sold in Canada (Al Naggar et al., 2015b). Due to importance of bees in Saskatchewan's farming industry and concerns over loss, a study of multiple colonies, was conducted to investigate whether or not NIs could be detected in bees and hive products. This information should help to understand the potential effects NIs may have on honey bee colonies in the Prairies.

2. Materials and methods

2.1. Sample collection and handling

Honey, pollen and worker bees' were collected from 7 independent apiaries located within a 30 km radius in central Saskatchewan (Fig. 1). Assuming a foraging range of 10 km from the hives studied, canola, alfalfa, wildflowers dandelions and willows made up the primary sources of nectar and pollen available to the bees in this study. Three, two-storey colonies were randomly selected at each apiary (~40 colonies at each location). Samples were collected in late August 2013, when flow of nectar was ending and populations of forager bees were largest. Samples from hives were stored in a cooler during transport to the University of Saskatchewan where they were kept frozen at −20 °C until extracted. Honey was collected directly off an open comb into 50 mL polypropylene falcon tubes, while pollen was collected by cutting 6 cm^2 piece of comb containing stored pollen using a disposable plastic knife and placed in 15 mL Falcon tube. Bees were collected from the wall farthest from the entrance because those bees would tend to be mostly foragers or older workers who would be expected to have potentially accumulated the greatest concentrations of pesticide residues from foraging as well as consuming hive products. Worker bees were carefully brushed directly into disposable polyethylene bags (Al Naggar et al., 2013a,b).

2.2. Standards and reagents

Certified analytical reference standards of seven NIs, clothianidin, imidaclopid, thiamethoxam, thiacloprid, flonicamid, acetamiprid, nitenpyram and mass labeled d3-imidacloprid and d4-clothianidin were purchased from (Sigma Aldrich, Ontario, Ca). Stock standards for use in calibration and determination of recovery were made up in methanol (MeOH). All solvents used in this study including nanopure water were HPLC grade or better and the Falcon tubes were from Fisher Scientific. Extraction materials magnesium sulfate, (MgSO4), sodium chloride (NaCl), trisodium citrate, disodium hydrogen citrate, and primary secondary amine (PSA) were purchased from (Sigma Aldrich, Ontario, Ca).

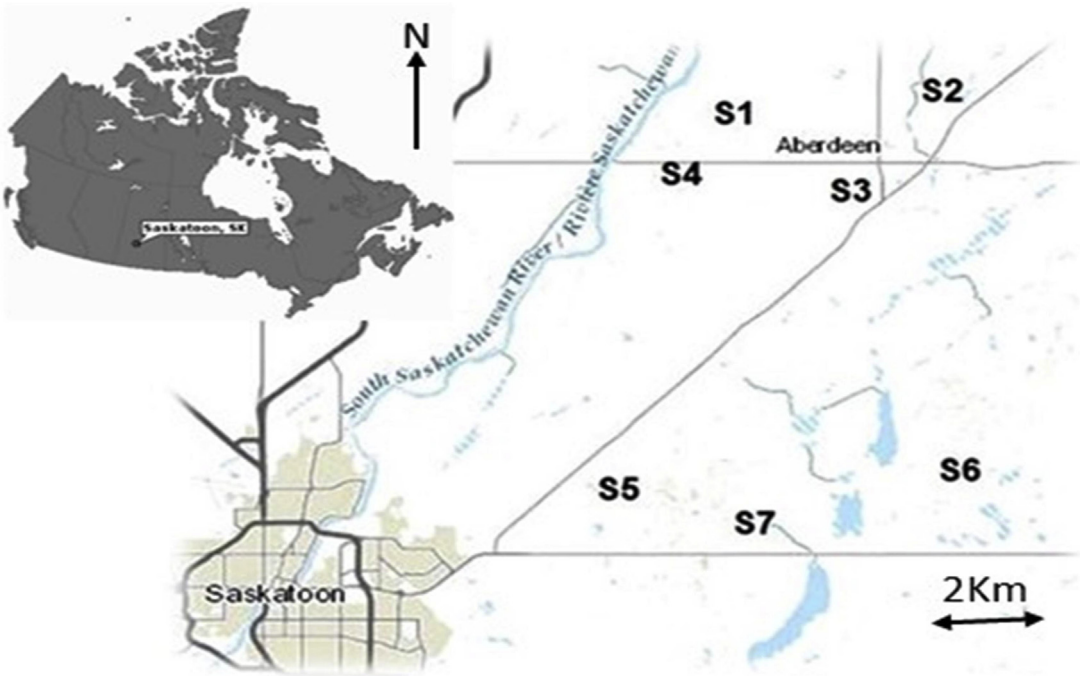

Fig. 1. Locations where samples were collected.

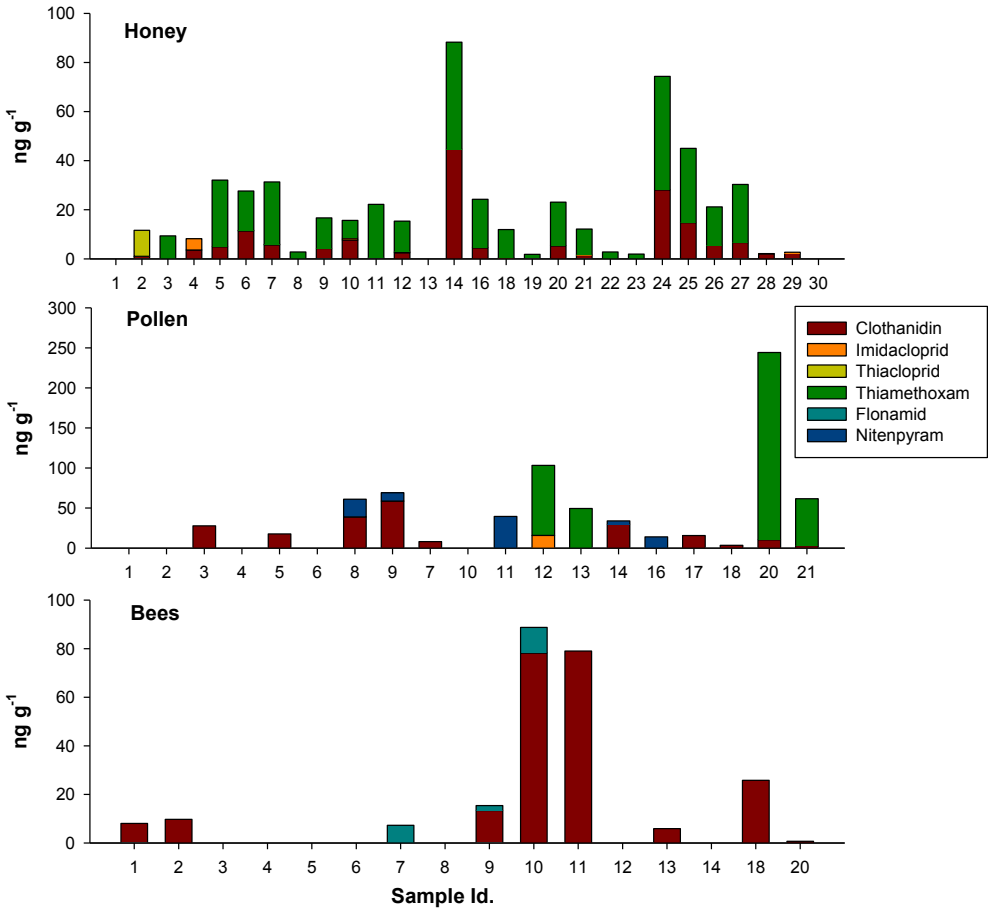

Fig. 2. Concentrations of untransformed active ingredient neonicotinoids (NIs) in honey, pollen and bees in Saskatchewan, Canada.

2.3. Extraction and cleanup

Samples were brought to room temperature and extracted by use of a modified QuEChRS method. Bees (~5 g) and pollen, (~2 g) having been extracted from the comb were weighed and homogenized by use of a pre-cleaned, glass pestle and mortar with 1–2 g of baked $NaSO_4$, followed by transfer to 50-mL Falcon tubes. Honey (~5 g) was weighed directly into 50 mL falcon tubes. Samples were fortified with 20 ng d3-imidacloprid surrogate standard (SS) and 10 mL of nanopure water introduced. Sample was vortexed for 1 min and shaken for 20 min, after which 10 mL of acetonitrile (ACN), was added and the sample vortexed and shaken again.

In 15-mL Falcon tubes 4 g $MgSO_4$, 1 g NaCL, 1 g trisodium citrate and 0.5 g disodium hydrogen citrate was prepared. A second Falcon tube containing 900 mg $MgSO_4$ 150 mg primary secondary amine (PSA) for use later was also prepared. After the second shaking, the mix of 4 compounds was added to the 50 mL tube and samples were shaken for 15 min and centrifuged at 3000 g for 5 min. Eight mL of the upper solvent (ACN) was pipetted to the second prepared 15-mL falcon tube. This was then vortexed and shaken for 15 min before centrifuging at 3000 g for 5 min, 6 mL of the supernatant was passed through a syringe filter (13 mm ø, 2 μm nylon syringe filter), to a clean 15-mL falcon tube and placed under N_2 to dryness. Samples were reconstituted in 200 uL ACN containing 50 ng mL^{-1} IS (d4-clothanidin), and analyzed by LC–MS/MS.

2.4. Identification and quantification

Analytes were separated by HPLC (Agilent) with a phenomonex C18 column (150 mm × 2.1 mm i.d. 1.8 μm particle size) and detected by MS/MS (AB Sciex API 3000) operating in MRM in negative mode with electrospray source. LC was run in gradient mode at 300 μl min^{-1} with mobile phases of (A) water/methanol (95:5) and methanol/water (95:5) both with 5 mM ammonium formate with 0.1% formic acid. Gradient started at 95% A holding for 3 min, before linear increase to 60% A in 12 min and a gradient to 95% B at 15 min holding for 3 min before returning to initial conditions for 8 min. MS source was at 350 °C, with nebulizer, curtain gas and collision gas at 13, 9 and 8 arbitrary units (AU) respectively, ion spray voltage was at 3500 eV. Untransformed, active ingredient NIs were quantified by comparison to by a 7 point calibration curve from 1 to 300 ng mL^{-1}, using retention time and multiple daughter ions, metabolites of imidaclopid, (See SI Table 1 for ions and LOQ values). Metabolites of imidacloprid were selected in this study based on the widespread use in the region SI Tables 2 and 3. Dinitrofuran and its metabolites were also included though authentic standards were not used in this study.

2.5. Quality control and assurance

Methods for extraction were developed and tested initially in Honey matrix. The three standard extraction methods for QuEChERS: (1) non-buffered 4 g $MgSO_4$, and 1 g NaCl; (2) AOAC method 2007.01 6 g $MgSO_4$ and 1.5 g NaAc; and (3) EN Extraction salts 4 g $MgSO_4$, 1 g NaCl, 1 g trisodium Citrate and 0.5 g disodium hydrogen citrate were evaluated, (Waters, 2011; Diez et al., 2006). Chauzat's method with a Chem Elut column (2006) and a C18 column were also tried. Based on these initial tests of 6 samples per method (3 spiked, 3 non spiked), the EN Extraction method gave >70% recovery. EN extraction method was further tested at a range of concentrations and blanks, and thus used.

Extraction blanks for honey used samples warmed to 80 °C for 5 h and gave no or less than the signal to noise ratio set as 3 times the baseline. Bee and pollen blanks were based on $NaSO_4$ media spiked and non-spiked ran alongside samples. Given the low mass of bees and pollen available in this study spikes of sample media was not possible. The $NaSO_4$ extractions would highlight any contaminants and variability within the extraction. During a standard instrument run ACN blanks and standards were ran every 6 samples to test for signal degradation and contamination.

Imidacloprid, acetamiprid, thiacloprid, thiamethoxam, flonicamid and nitenpyram were identified and quantified based on multiple paired parent and transition ions, (SI Table 1). Metabolites were calculated using external, calibration curves so results should be treated as estimates rather than actual concentrations. Dinitrofuran and its metabolites were also identified but without authenticated standards these will not be discussed. Using multiple paired ions positive identification was limited to finding all daughter ions and having a calculated concentration of <10% variability between pairs.

Mean recovery of d3-imidacloprid from pollen was 61% (median 60%), 51% (median 55%) from bees, and 68% (median 68%) from honey, with initial test recoveries of the EN method at 80%, across multiple concentration ranges (2–300 ng mL^{-1}). Two samples of honey contained matrix interferences leading to IS and SS recovery of <10% and far outside the range of other samples so are excluded from this study.

3. Results and discussion

In total 26 samples of honey, 19 of pollen and 16 of worker bees were analyzed. Of untransformed active ingredient NIs, clothianidin was the most commonly detected, found in 86% of honey, 58% of pollen and 56% of bee samples. Thiamethoxam was also commonly found in honey while nitenpyram was found only in pollen. Acetamiprid was not detected in any sample and thiacloprid was observed in only one sample. The percentage of positive samples were greater in this study than those reported previously in the review by Blacquière et al. (2012), where clothianidin was mostly less than the LOD in most studies and imidaclorpid was the most commonly detected NI. Concentrations of NIs were comparable to those found in other studies (Table 1).

Since in the region from which samples were collected, canola was one of the predominant crops providing nectar and pollen sources for foraging bees, detection of clothianidin and its precursor thiamethoxam as the most common compounds is not surprising. Both of these NIs are commonly used to treat seeds of canola (Cutler and Scott-Dupree, 2007). Since 2002, in the UK and Japan, there has been a significant increase in use of thiamethoxam compared to other NIs, (Simon-Delso et al., 2015). Consequently, the worldwide sales of thiamethoxam reached US $1 billion in 2011 (Syngenta, 2012), and US $1.1 billion in 2012 (Syngenta, 2013). Since samples were collected late in the season some breakdown, due to metabolism of thiamethoxam to clothianidin that occurs in insects, soils and plants, was likely, which would have resulted in the infrequent rate of detection of thiamethoxam in bees (n = 1) and pollen (n = 4) (Nauen et al., 2001) (see Fig. 2).

3.1. Transformation products of imidaclopid

Since no authentic standards were available for metabolites or transformation products of imidaclopid, quantification of was semi-quantitative. Transformation products of imidaclorpid, (imidaclorpid-olefin, 5-hydroxy, Urea, desnitro-HCL and 6-chlorotonic acid), were chosen based on use of imidacloprid in Canada (SI Tables 2 and 3). In honey, the mean concentration of imidaclorpid was 0.8 ng g^{-1} wm, while metabolites imidaclorpid-olefin and 5-hydroxy were estimated to 5.6 and 21.1 ng g^{-1}, wm respectively. Greater concentrations of transformation products, relative to imidaclorpid, indicate that greater focus should be placed on

Table 1
Concentrations of untransformed, active ingredient, neonicotinoids (NIs) in honey, pollen and bees reported previously.

Compound	Pollen	Bee	Honey	Ref.
	ng g^{-1}			
Clothianidin	2.6[a]	nd	0.9[a]	Cutler and Scott-Dupree (2007)
	2.9 (healthy)	n.d (healthy)		Krupke et al., 2012
	10.7 (impacted)	3.8–13.3 (dead/dying)		
	0.1–15	1–40		Pistorius et al. (2009)
Acetamiprid	57 (14–134)	nd	nd	Mullin et al. (2010)
Imidacloprid	912[a]	nd	2[a]	Kamel (2010)
	20.5 (6.2–206.0)			Mullin et al. (2010)
Thiacloprid	115[a]	nd	33[a]	Frazier et al. (2008)
	5.9 (1.9–7.8)			Mullin et al. (2010)
Thiamethoxam	53	nd	nd	Mullin et al. (2010)
	6.2 (healthy)	n.d		Krupke et al., 2012
	20.4 (impacted)			
Imidacloprid 5-Hydroxy	152			Mullin et al. (2010)
Imidacloprid olefin	554			Mullin et al. (2010)

[a] These are the upper concentrations.

metabolites when assessing risks of use of this insecticide (Fig. 3). Pathways of transformation of imidaclopid can be divided into two major processes; oxidative cleavage and hydroxylation (Klein, 1994, Thyssen and Machemer, 1999). During oxidative cleavage 6-chloronicotinic acid is the major product with further transformation possible. During hydroxylation, 5-hydroxy and olefin derivatives can be detected, (Klein, 1987). In studies of eggplant cabbage, oxidative cleavage was shown to occur (Mukherjee and Gopal, 2000), inducing greater toxicity to aphids (Nauen et al., 1998). In this study imidaclopid-olefin and imidaclopid 5-hydroxy were detected in the majority of samples of honey, but products of oxidation were <LOQ. The metabolite 6-chloronicotinic acid was detected in 2 samples of bees which might indicate some breakdown of imidaclopid or uptake of metabolites from plants. Other breakdown products of imidaclopid were sporadically detected in both bees and pollen (Fig. 3). Given the limited number of detections in pollen and bees compared to honey it was not possible to find evidence that there is transfer between bees and dietary exposure.

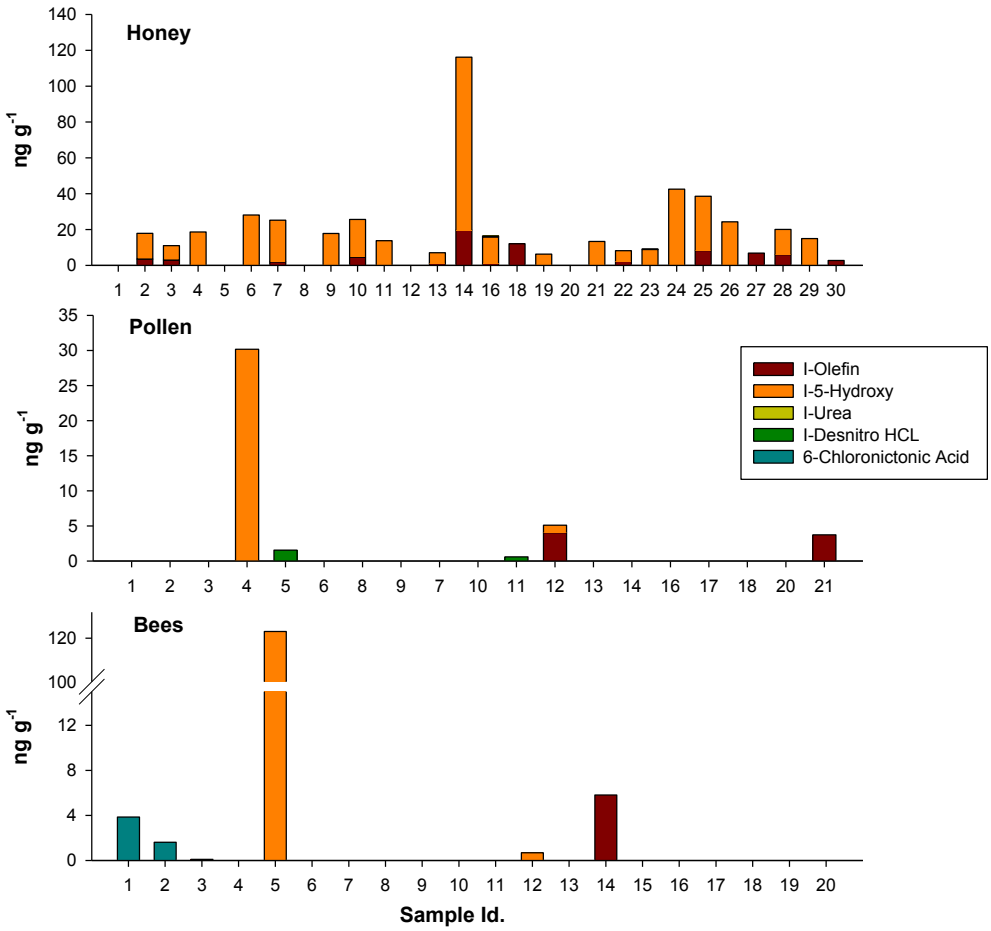

Fig. 3. Metabolites determined within honey, pollen and bees of samples within the Saskatoon region in Saskatchewan, Canada.

3.2. Health risks to bees

There are several ways to estimate risks of adverse effects and in this paper a comprehensive assessment of potential effects of NIs on the honey bee are not presented. The assessment presented here is based on lethality and meant to put concentrations observed in bees and bee products into perspective. In particular, effects on behavior and or chronic effects on immune function were not considered. Such assessments have been done in greater detail in other studies of exposures of bees in other areas (Blacquiere et al., 2012; Krupke et al., 2012; Fairbrother et al., 2014; Goulson, 2013). Using average LD_{50} values for lethality this section aims to evaluate whether concentrations of NIs in the various matrices investigated might exceed thresholds for lethality. Concentrations presented in Table 2 are the detected values only. For example, the mean concentration of thiamethoxam was 0.19 ng bee^{-1} from consumption of pollen. However, this result included only 26% of the total number of samples. Thus, the mean concentration to which bees would be expected to be exposed would have been less than the "maximum" mean calculated without those samples that contained concentrations that were less than the LOQ.

LD_{50}s of NIs for worker, honey bees have been determined. For untransformed NIs, acetamiprid has relatively less oral toxicity (7000–14,000 ng bee^{-1}), while clothianidin has greater toxic potency (2.5–44 ng bee^{-1}) depending on the study, (EU Commission, 2004; Bonmatin., 2015; Iwasa et al., 2004; Laurino et al., 2013; Kamel, 2010; Bonmatin et al., 2015). Metabolites of imidaclorpid have greater toxic potencies with imidaclorpid-olefin being more toxic than the untransformed (parent) compound (mean concentrations of 32 compared to 116 ng bee^{-1} respectively; EU Commission, 2004; Laurino et al., 2013; Suchail et al., 2000; Kamel, 2010; DEFRA, 2007, 2009). LD_{50} values for these insecticides are affected by a range of abiotic and biotic factors and there are a range of LD_{50} values available for each (SI Table 4). For imidacloprid, values of 5–500 ng bee^{-1} have been reported and much depends on the design of the study in question, the way that pesticides are introduced and the individual bee.

Based on the average mass of honey bee workers of 90 mg, concentrations in individual bees were calculated (Moritz and Southwick, 1992). The mean concentration of clothianidin for samples in which it was detected, 2.5 ng bee^{-1} was near the estimated LD_{50} 2.5 and 3.5 ng bee^{-1}, estimated by Bonmatin et al (2007) and Kamel (2010), respectively, but less than LD_{50}s estimated by others (SI Table 4). For all other untransformed, NIs detected, concentrations found in bees were orders of magnitude less than LD_{50}s (Table 2, SI Table 4). Said another way, the margin of exposure (MOE) were ranged from 1×10^4 to 2×10^3 for Flonamidid.

In winter it is estimated that a hive will consume 13.6 kg of honey and a typical hive will have approximately 10,000 worker bees that will live for 4–9 months (Moritz and Southwick, 1992). Taking these factors into account and assuming no loss of NIs or breakdown in the stored honey, uptake of honey during winter was estimated for individual worker bees (Table 2; winter uptake of worker). In this very conservative scenario, worker bees were predicted to be exposed to a mass of NI that would in a single dose exceed the lower threshold LD_{50} values for thiamethoxam and clothianidin. This would be a worst case scenario that would not be expected to be achieved, but it does provide an upper bound on potential exposures of overwintering workers.

It is estimated that a worker honey bee will consume ~2.0 mg of pollen per day over winter. The actual mass of pollen consumed depends on the source (USEPA, 2014). Based on what was detected in pollen samples and using values of oral LD_{50} values for adult bees, it is concluded that daily consumption of pollen is not a major source of these contaminants to adult bees during summer. However, taken over an estimated winter period of 150 days in Saskatchewan total uptake exceeds minimum estimated LD50s for some NIs. In a 3 year study of imidacloprid dosed pollen on bee health concentrations >20 ng g^{-1} were shown to have negative effects on honey bee colonies in this study concentrations were below this (<14.1 ng g^{-1}) (Dively et al., 2015).

While there is uncertainty in relative sensitivities of bee larvae, it is unlikely that consumption of NIs in pollen would exceed a critical threshold for lethality given the small mass of pollen consumed, which has been determined to be 1.8 and 3.6 mg day^{-1} for larvae of drone and worker bees, respectively and the short period of growth. Other worker bees involved in activities such as cell cleaning and capping do consume greater masses of pollen (up to 12 mg day^{-1}) in summer and may be exposed to NIs stored in pollen, but their lifespan is shorter ~45 days, limiting overall risk (USEPA, 2014).

4. Conclusion

Concentrations of NIs in most samples of pollen and honey did not exceed or even come close to the oral LD_{50} values for bees. However, unlike the results of previous studies the proportion of hives where NIs were detected was greater and clothianidin was the most detected NI in honey. It is concluded that direct exposure and or dietary exposure to NIs from honey and pollen by bees over winter suggested that, in some hives, consumption of honey and

Table 2
Exposures of bees to neonicotinoids (NIs) in the diet of bees and measured in bees of Saskatchewan, Canada, based on detected NIs. The concentration lethal to 50% of bees is also given.

Compound	LD_{50}	Bees	Honey	Pollen	Bee	Winter exposure	
						Honey	Pollen
	ng bee^{-1}	% Detection				ng bee^{-1}	
Clothianidin	18.0 (2.5–44)	52.9	67.9	57.9	2.5, (0.1–7.1)	6.7, (0.7–20.0)	5.2 (<0.01–15.7)
Imidaclorpid	116 (3.7–490)	N.D.	32.1	5.3	N.D.	1.1, (0.2–6.2)	4.2
Thiacloprid	23,300 (12,600–38800)	N.D.	3.6	N.D.	N.D.	14.4	N.D.
Thiamethoxam	11.8 (3.5–30)	N.D.	75.0	21.1	N.D.	19.4, (2.5–41.1)	28.7 (13.2–62.5)
Flonamidid	>2000	17.6	N.D.	N.D.	0.6, (0.2–1.0)	N.D.	N.D.
Nitenpyram	80.5 (23–138)	N.D.	N.D.	26.3	N.D.	N.D.	4.48, (1.2–10.5)
Imidaclorpid Metabolites							
Imi olefin	32 (28–36)	11.8	46.4	10.5	0.3, (0.3–0.5)	6.0, (1.4–16.4)	1.0 (0.99–1.1)
Imi 5-hydroxy	153.5 (49–258)	11.8	71.4	10.5	5.6, (0.1–11.1)	21.4, (8.1–41.2)	28, (0.15–0.41)
Imi urea	>1000	N.D.	7.1	N.D.	N.D.	N.D.	N.D.
Imi-desnitro HCL	>1000	N.D.	3.6	10.5	N.D.	N.D.	0.28 (0.15–0.41)
6-Chloronictonic ACID	>1000	11.8	N.D.	N.D.	0.2, (0.1–0.3)	N.D.	N.D.

pollen during over-wintering might have adverse effects on bees in Saskatchewan. However, a further study is required across a wider area of the Prairies of Canada to determine if the concentrations determined are limited to the sample region or more representative of the whole region. Further investigation should also include a greater range of metabolites particularly those for clothianidin. One uncertainty is what proportion of worker bees might have been killed by exposure to NIs. For instance, if a worker was exposed to an acutely lethal dose of NIs, it would not have been measured when workers were sampled. This might bias the overall average concentrations measured in worker bees. Since NIs would be expected to have the same mechanism of toxic action, but with different potencies, another uncertainty not addressed in this initial assessment of risk was that there might be joint actions due to exposure to multiple NIs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2015.10.135.

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