Environmental Impacts of Proposed Management Options

Neonicotinoid Pesticides Cause Mass Fatalities of Native Bumble Bees: A Case Study From Wilsonville, Oregon, United States

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Abstract

In June of 2013 an application of dinotefuran on an ornamental planting of European linden trees (Tilia cordata Mill. [Malvales: Malvaceae]) in a shopping mall parking lot in Wilsonville, Oregon provoked the largest documented pesticide kill of bumble bees in North America. Based on geographic information systems and population genetic analysis, we estimate that between 45,830 and 107,470 bumble bees originating from between 289 and 596 colonies were killed during this event. Dinotefuran is a neonicotinoid that is highly effective in exterminating and/or harming target pest insects and non-target beneficial insects. Analysis to detect the concentration of pesticides in flowers that received foliar application revealed that the minimum reported dinotefuran concentration of a sampled T. cordata flower was 7.4 ppm, or in excess of 737% above the LC50 of the beneficial pollinator, the honey bee (Apis mellifera Linnaeus, 1758 [Hymenoptera: Apidae]). Furthermore, sampled Vosnesensky bumble bees (Bombus vosnesenskii Radoskowski, 1862 [Hymenoptera: Apidae]) were found to have an average dinotefuran concentration of 0.92 ppm at the time of death, which exceeds the maximum LC50 of A. mellifera (0.884 ppm). Our study underscores the lethal impact of the neonicotinoid pesticide dinotefuran on pollinating insect populations in a suburban environment. To our knowledge, the documentation and impact of pesticide kills on wild populations of beneficial insects has not been widely reported in the scientific literature. It is likely that the vast majority of mass pesticide kills of beneficial insects across other environments go unnoticed and unreported.

Key words: pesticide kill, dinotefuran, colony number estimation, population genetic analysis, LC50

The decline of beneficial insects, including pollinators, is a conservation issue of considerable economic and ecological importance (Memmott et al. 2004). Bumble bees (Bombus spp., [Hymenoptera: Apidae]) are important pollinators throughout temperate regions, particularly in the Northern Hemisphere. Their long tongues, ability to fly in low temperatures and inclement weather, as well as their ability to buzz pollinate (Heinrich 2004) make them second only to the honey bee as contributors to the multi-billion dollar agricultural industry (Delaplane and Mayer 2000, Kremen et al. 2002, Klein et al. 2007, Gallai et al. 2009). Bumble bees are also essential pollinators in wildlands and natural areas as generalist pollinators of many plant families (Goulson 2010). However, there have been alarming reports of bumble bee population declines from multiple continents (Williams 1982, Goulson et al. 2008, Williams and Osborne 2009, Williams et al. 2009, Martins and Melo 2010, Cameron et al. 2011, Cameron and Sadd 2020), including North America (Colla and Packer 2008, Grixti et al. 2009, Cameron et al. 2011, Colla et al. 2012, Hatfield et al. 2015).

The ultimate cause of bumble bee and other native bee declines continues to be investigated, although many factors are contributing (Thorp et al. 2003, Colla et al. 2006, Williams et al. 2009, Cameron et al. 2011, Koch and Strange 2012, Cameron and Sadd 2020). While land use change and habitat fragmentation are likely contributors to decreasing populations in some species (Williams and...
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Osborne 2009, Potts et al. 2010, Cameron and Sadd 2020), other factors include: pathogen infection (Thorp et al. 2003, Colla et al. 2006, Williams et al. 2009, Cameron et al. 2011, Koch and Strange 2012, Cameron and Sadd 2020), pesticide use (Desneux et al. 2007; Laycock et al. 2012, 2013; Whitehorn et al. 2012; Fauser-Misslin et al. 2013; Baron et al. 2014; Cameron and Sadd 2020), and climate change (Williams et al. 2009, Kerr et al. 2015, Sirois-Delisle and Kerr 2018, Cameron and Sadd 2020).

Insecticides, particularly systemic insecticides including neonicotinoids, are increasingly implicated in bee and other wildlife declines (van Lexmond et al. 2014; Pisa et al. 2015, 2017; Eng et al. 2019). The spectrum of effects on bees ranges from sublethal (i.e., not causing immediate mortality, rather behavioral and/or biological effects that reduce colony fecundity) to lethal depending on the dose and length of exposure (Whitehorn et al. 2009, 2012, 2013; Cresswell et al. 2013; Laycock and Cresswell 2013; Gill and Raine 2014; Laycock et al. 2014; Wood and Goulson 2017; Crall et al. 2018). There are four nitroguanidine neonicotinoids that are particularly toxic to honey bees: imidicloprid, thiamethoxam, dinotefuran, and clothianidin (Iwasa et al. 2004). Risk assessments to pollinators typically identify LD$_{50}$ (lethal dose to 50% of the test population) of honey bees (Apis mellifera Linnaeus, 1758 [Hymenoptera: Apidae]), which appear to have a higher threshold than bumble bees for acute lethal and sublethal effects of nitroguanidine neonicotinoids (Arena and Sgolastra 2014, Hopwood et al. 2016).

Dinotefuran is classified as highly toxic (acute LD$_{50} <$ 2μg/bee) to honey bees with an acute contact LD$_{50}$ of 0.024–0.061 μg [LC$_{50}$ = 0.923–2.23 ppm (Iwasa et al. 2004, EPA 2004) and an acute oral LD$_{50}$ of 0.0076–0.023 μg [LC$_{50}$ = 0.292–0.885 ppm; EPA 2004]). Dinotefuran, first introduced in 2005, was developed for the treatment of a broad range of insect pests for a diversity of commercial and residential uses on leafy plant material (EPA 2004). However, it is the least used of the four highly toxic nitroguanidine neonicotinoid insecticides (Fig. 1) (Baker and Stone 2015).

Acute toxic exposure to insecticides is often the focus of EPA pesticide assessments (EPA 2014). However, delayed toxicity and sublethal effects are a significant concern, especially for nitroguanidine neonicotinoids, which are long-lived in plants, especially woody tissue (Mach et al. 2018). Multiple studies have shown that nitroguanidine neonicotinoids have sublethal effects in bumble bees. These effects include reduced brood production (Elston et al. 2013), immune suppression (Fauser-Misslin et al. 2013), reduced food consumption (Elston et al. 2013), impaired foraging (Feltham et al. 2014), impaired nest initiation by queens (Leza et al. 2018) and altering behavior and thermoregulation (Crall et al. 2018). The sublethal effects of dinotefuran on bees are not well studied (Hopwood et al. 2016), but the effects of chemicals with similar modes of action and levels of toxicity have been documented (Iwasa et al. 2004; Pisa et al. 2015, 2017). Sublethal effects in bumble bees from imidicloprid, thiamethoxam, and clothianidin have been detected at concentrations between 0.0002 – 0.01 ppm (Fischer et al. 2014, Mommaerts et al. 2010, Elston et al. 2013).

While these pesticides are widely used in agricultural and landscape pest control, the toxicity to non-target organisms has raised environmental concerns (Pisa et al. 2014, Wood and Goulson 2017). Compounding the concerns, human error in pesticide applications has been known to lead to unintended negative impacts including human injury, wildlife death, and bee kills (Barnett et al. 2007). Bee kills in domesticated honey bee hives have been well documented (Barnett et al. 2007), but large-scale impacts on wild bees are poorly studied, in large part because they are rarely observed and probably mostly occur away from most human observation.

In June of 2013 an application of dinotefuran on an ornamental planting of European linden trees (Tilia cordata Mill. [Malvales: Malvaceae]) in a suburban shopping mall parking lot in Wilsonville, Oregon provoked the largest documented pesticide kill of bumble bees in North America (Fig. 2). In this study, we use ground

![Fig. 1. Nitroguanidine neonicotinoid use in the United States by year in kg (Left: EPest-low, Right: Epest high) (Data: Baker and Stone 2015; Baker 2015, 2016; Thelin and Stone 2013).](attachment://image.png)
surveys and microsatellite markers to estimate the number of Vosnesensky bumble bees (Bombus vosnesenskii Radoskowski, 1862 [Hymenoptera: Apidae]) that were killed and provide evidence that the cause of death was dinotefuran contaminated T. cordata flowers. We present information on the dinotefuran application rates that were applied to the T. cordata and the residue levels of neonicotinoids on sampled T. cordata flowers and tissues of dead B. vosnesenskii. Given the negative impact of the legal dinotefuran application on pollinating insects in a commercial setting, we discuss best pesticide and landscape management practices.

Methods

Description of Wilsonville, Oregon, USA Pesticide Incident

On 17 June 2013 the author (R. Hatfield) received phone calls from concerned community members regarding the presence of dead bees under trees in a parking lot of a commercial retail facility in Wilsonville, OR, USA (45.333375, -122.767160). We visited the commercial retail facility and discovered vast numbers of dead bumble bees (Bombus spp.) underneath T. cordata (Fig. 2). We identified the overwhelming majority of bumble bees to the species B. vosnesenskii. However, additional bumble bee species lethally impacted by the poisoning event included the fuzzy-horned bumble bee (Bombus mixtus Cresson 1878 [Hymenoptera: Apidae]), and Bombus caliginosus Frison 1927 (Hymenoptera: Apidae). We immediately alerted the Oregon Department of Agriculture (ODA), which initiated a pesticide investigation. From a preliminary investigation we learned that on 15 June 2013 a landscape maintenance company treated approximately 55 T. cordata while in bloom for aphids (Aphidoidea) and root weevils (Curculionidae) with dinotefuran at the retail facility in Wilsonville. We learned later that the maintenance company used two separate treatments based on trees’ location to parked cars (Fig. 3). The applicators treated approximately 46 trees with a foliar application at a rate of 6 oz per 100 gal (46.87 ml/liter) and 9 trees with a soil drench application rate of 1/3rd oz/in (3.85 ml/cm) DBH in 4 gallons (15.14 liter) of water; no adjuvants were mixed into the Safari application and both application rates were within the recommended concentrations on the product label (ODA 2014a). In a foliar application, the product is sprayed directly onto the vegetation and flowers at the chosen concentration. The insecticide is then on the tissues of the plant, but is also absorbed by the plant, and then continues to be expressed into the nectar that the tree produces. A soil drench relies on the water solubility and systemic nature of neonicotinoids. Typically, a trench is dug around the tree, and then the treatment is poured onto the ground based on the size of the diameter of the tree at breast height (DBH). The roots of the tree then absorb the insecticide and it is delivered to the remainder of the tree (including pollen and nectar in flowers) via the xylem (Cloyd et al. 2011).

Pesticide Analysis

ODA collected Bombus and T. cordata tissue and conducted an analysis to detect and quantify pesticides in tissue samples. ODA collected 303 g of dead bumble bees throughout the affected area and a composite of T. cordata branches (200–250 mm long branch tips) from trees north of the commercial retail building (ODA 2014a). All samples were collected following standard ODA protocols and processed in ODA laboratory facilities in Salem, OR (ODA 2014a). The samples were not collected as part of a systematic study, and it is unknown whether samples represent the peak of pesticide concentration in bee or plant tissues. On 11 July 2013 ODA returned to the site and collected additional flower, leaf, and stem samples from treated T. cordata to test for differences between the soil drench and foliar application treatment types. ODA also returned in 2014 to collect additional samples of T. cordata to test for residual concentrations of dinotefuran.

While pesticide LD$_{50}$ for honey bees are reported in μg/bee (EPA 2004) analytical pesticide tests for contamination are reported in ppm. We used Equation 1 to convert LD$_{50}$ ranges to estimated LC$_{50}$ (lethal concentration to 50% of the test population) (Fischer and Chalmers 2007) to make them comparable to the concentrations found during the tissue analysis conducted by ODA (in ppm). The conversion here is imprecise due to the unknown amount of liquid fed to individual treated bees, nor the precise concentration of sugar solution during testing.

$$LC_{50} \approx \frac{LD_{50} \text{ in } \frac{\mu g}{\text{bee}} \times 1000}{\text{est. amt. of solution fed to bees}}$$

Bumble Bee Density Estimation

Upon arrival at the site, we took several photographs of dead bumble bees on the ground (Fig. 2 and Supp Fig. 1C-I [online only]). To estimate the number of dead bumble bees killed in the
pesticide incident, we combined the photographs with georeferenced aerial photographs of the site in ArcMAP (ESRI 2011) and aerial photography from World Imagery Base Layer (ESRI 2010) to measure two parameters: 1) total area covered with bumble bee carcasses using tree canopy circumference and 2) average density of bumble bee carcasses per meter measured by counting carcasses in photographs and using the area of a parking lot space at this location to generate the area covered in carcasses (see Figs. 2 and 3, and Supp Fig. 1C–I [online only]). Our density estimate is from one day of collection (17 June 2013) and there were ODA reports that the parking lot had been swept prior to our visit on 17 June 2013 (ODA 2014a). In addition, birds, ants (Formicidae) or other scavengers may have removed an unknown number of the dead bees (Supp Fig. 1A [online only]). On 17–19 June 2013 bumble bees were observed foraging on *T. cordata* flowers and dying after photographs were taken until the trees were covered by the ODA and the City of Wilsonville with large mesh canopies made from landscaping fabric on 20 June 2013 (Supp Fig. 1B [online only]). Despite state and city efforts to create a physical barrier between the poisoned *T. cordata* and wild insects, bumble bees continued to get into the netting and visit *T. cordata* flowers after they were covered (R. Hatfield pers. obs.). Considering the dynamics of this pesticide poisoning event, we suspect that actual bumble bee mortality exceeded the estimates presented in this study. In estimating the total area of land covered by bumble bee carcasses we used aerial photography from 2010 and used canopy diameter as an estimate for area covered. Since the event was nearly three years after the aerial photography used to estimate canopy cover (2010), and bumble bee carcasses extended well beyond the edge of the canopy, we suspect that our estimate on the number of bumble bee carcasses across the incident area is conservative.

**Colony Number Estimates**

To estimate the number of bumble bee colonies associated with the pesticide-linked fatalities we revisited the site on 16 July 2013. By that date, all dinotefuran treated trees had been netted to prevent further bee fatalities. We found dead bumble bees inside the netting and collected samples from 19 *T. cordata* spatially distributed throughout the affected area (Fig. 3). The number of bumble bees collected from individual *T. cordata* varied from 3 to 36 individuals. Bumble bees were collected into dry 50 ml centrifuge tubes by the tree, labeled, and then shipped to the USDA-ARS Pollinating Insect

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Fig. 3. Map of the study area. Polygon size represents the approximate canopy extent in 2013. White trees were treated with a foliar application of dinotefuran, gray trees were treated with a soil drench application of dinotefuran. We collected bumble bees for genetic analysis from trees with hatched overlay, and we took the density estimate photos under the tree with the cross-hatch overlay. Imagery: ESRI, Maxar, Earthstar Geographics, CNES/Airbus DS, and the GIS User Community; Treatment Data: ODA 2014a.
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Biology, Management, Systematics Research Unit in Logan, UT, USA for genetic analysis. Most of the specimens were B. vosnesenskii and only specimens of that species were included in our subsequent analysis of colony numbers.

We extracted DNA by macerating leg tissue from each specimen in 150 ul of 5% Chelex solution and 5 ul of Proteinase K (Strange et al. 2009). Samples were incubated for 1 h at 55°C, 15 min at 99°C, 1 min at 37°C, and 15 min at 99°C. Extracted DNA was then screened at 11 microsatellite loci: BTMS0044, B124, B96, B126, BT30, BTMS0083, BTMS0059, BTMS0066, BT10, BTMS0062, and BTMS0086 (Estoup et al. 1995, 1996; Reber Funk et al. 2006; Stolle et al. 2009). Polymerase chain reaction (PCR) for each locus were multiplexed in a final reaction volume of 10 ul, containing approximately 1 ul of extracted DNA, 1× Promega (Madison, WI) reaction buffer, 0.6 mM dNTP mixture, 0.2 – 0.4 μM primer, 1.4 mM MgCl₂, 0.001 mg BSA, 0.4 units Taq polymerase (Promega, Madison, WI) and ddH₂O to fill to volume. The thermal cycler for PCR began with denaturation at 95°C for 7 min, 30 cycles of 95°C for 30 s, annealing temperature 53°C for 1 min 30 sec, 72°C for 30 s, and a final extension at 72°C. DNA amplifications were performed with fluorescent 5' dye-labeled primers and separated on an Applied Biosystems 3730xl automated sequencer. Alleles were scored manually using GeneMapperTM v5.0 software (Applied Biosystems). Samples with >9 loci scored per individual were included in the colony and population genetic analysis.

Microsatellite loci were investigated for allelic dropout and null alleles with Microcheck2 (Van Oosterhout et al. 2004). Deviations from Hardy–Weinberg and linkage equilibrium were examined with GenePop v4.2 (Raymond and Rousset 1995, Rousset 2008) with 1000 dememorizations, 100 batches, and 1000 iterations per batch using the Markov Chain approximation for the exact tests and likelihood-ratio tests, respectively. To estimate sibship and colony assignment among the B. vosnesenskii collected we employed the maximum likelihood algorithm in COLONY v2.0 (Jones and Wang 2010). In our colony assignment analysis, we assumed a genotyping error rate of 0.001 based on previous studies of B. vosnesenskii (Rao and Strange 2012, Jha and Kremen 2013, Jha 2015). To examine the relationship between individuals surveyed, colony number, and the number of sibships we randomly sampled individuals at 26 intervals between 1 and the total evidence set (n = 492). For each iteration, we used COLONY to determine how many colonies and sibships were associated with the subsampling effort.

Preliminary analysis of the genetic data found that we were unable to capture the total number of colonies due to low sampling of individuals. Thus, to predict how many colonies extend beyond our sampling effort, we elected to fit our data to a 3-parameter asymptotic exponential function described in Equations 2 and 3.

\[
y = a - be^{-cx}
\]  

(2)

where, \(a\) is the horizontal asymptote on the right-hand side, \(b\) is \(a - R_0\) (\(R_0\) = the response \(y\) when \(x\) is zero), and \(c\) is the rate constant.

\[
c = \frac{\log((a-y)/b)}{x}
\]  

(3)

Rather than providing an initial estimate for the model parameters \(a\), \(b\), and \(c\), we elected to use the self-starting non-linear functions available in \(R\) to minimize model failure (Crawley 2007). We used the function \(SSasympto\) in the \texttt{stats} library to construct the model with subsampled full sibling colony estimates as the dependent variable, and the number of subsampled individuals as the independent variable. We used the function \texttt{predictNLS()} in the \texttt{propagate} library to calculate 95% prediction intervals of the nonlinear models for the supplied predictor values by using first-second-order Taylor expansion and Monte Carlo simulation (\(n = 100,000\)). The prediction interval is \([0, 5000]\). We used the function \texttt{confint2()} in the \texttt{nls tools} library to calculate the 95% confidence interval of the three model parameters. Finally, we used the program Capwire (Pennell et al. 2013) to produce a comparable estimate of undetected colonies in our dataset following approaches described with B. vosnesenskii genotype data elsewhere (Mola et al. 2020). With a similar aim to the approaches described above, we used Capwire to achieve a maximum likelihood estimate of colony abundance and 95% confidence interval using an Equal Capture Model (ECM) where all genotyped individuals are assumed to have an equal probability of being captured (Pennell et al. 2013).

Results

Pesticide Analysis

A composite of T. cordata stem, leaf, and flower samples collected on 17 June 2013 had an average dinotefuran concentration of 2.9 ppm [Method Detection Limit (MDL) = 0.004 ppm]. T. cordata samples collected on 1 July 2013 were differentiated by plant tissue and treatment type and varied significantly in dinotefuran concentration (Wilcox rank-sum test: \(W = 0, P = 0.03\)) (Table 1). Flowers from trees that were treated with a soil drench had dinotefuran concentrations that varied from 0.012 to 0.12 ppm (MDL = 0.004 ppm) (ODA 2014a). Dinotefuran concentration in flowers from trees that received a foliar application (46 of 55 trees) exceeded the LC₅₀ of A. mellifera by 800%–3,767% [acute oral LC₅₀ for A. mellifera = 0.292–0.885 ppm, measured concentrations trees treated with a foliar application—7.4–11.0 ppm—were at least 8× the high end (7.4 ppm/0.885 ppm) of the LC₅₀ and more than 37× the minimum (11.0 ppm/0.292 ppm) LC₅₀]. In our assessment, we found that the minimum reported dinotefuran concentration of the sampled T. cordata flower is 7.4 ppm and the maximum LC₅₀ for A. mellifera for dinotefuran is 0.884 ppm. Finally, the dinotefuran concentration in flowers found on T. cordata treated with soil drench was between 1%–41% of the LC₅₀.

The sampled bumble bees had a dinotefuran concentration of 0.92 ppm (MDL = 0.004 ppm) (Table 1). The concentration of dinotefuran was above the method detection level (MDL) of 0.004 ppm.

| Table 1. | Concentration of dinotefuran from lab results of an ODA investigation into a pesticide application on Tilia cordata in June 2013 |
|----------|---------------------------------------------------------------|
| Tissue tested | Application Method | 2013 Results (ppm) | 2014 Results (ppm) |
| Bombus spp. | Mixed | 0.93 | N/A |
| Tilia composite | Mixed | 2.9 | N/A |
| Tilia flower 1 | Foliar | 7.4 | Not Detected |
| Tilia flower 2 | Foliar | 11 | Not Detected |
| Tilia flower 3 | Drench | 0.12 | 0.076 |
| Tilia flower 4 | Drench | 0.012 | 0.024 |
| Tilia leaves 1 | Foliar | 5.4 | Not Detected |
| Tilia leaves 2 | Foliar | 3.8 | Not Detected |
| Tilia leaves 3 | Drench | 0.39 | 0.65 |
| Tilia leaves 4 | Drench | 0.97 | 0.63 |

All test results were conducted using a method detection level (MDL) of 0.004 ppm.
Bumble Bee Density Estimation

The total area covered with bumble bee carcasses under *T. cordata* treated with a foliar application (*n* = 46) was 1,725.3 m$^2$. The total area covered with bumble bee carcasses under *T. cordata* treated with a soil drench (*n* = 9) was 276.0 m$^2$ (Fig. 3). A photograph taken by R. Hatfield captured 48 dead bees in an area of approximately 0.3022 m$^2$ (Fig. 2B). Thus, based on a count of dead bumble bees from this photograph and the area estimated from a standard pencil, we estimated a density of 158 dead bees per m$^2$. However, Fig. 2A represents a photograph of a tree that experienced a foliar application, on the edge of the parking lot, so it likely experienced a higher than normal exposure to foraging bumble bees approaching the parking lot (assuming bees approached the parking lot from outside of the treated site perimeter). Therefore, to improve the estimate, we examined seven additional photographs of unique locations in the parking lot of the site (Supp Fig. 1C–I [online only]). We counted the number of bumble bee carcasses in each photo and used the approximate area of a parking lot space at this site to generate each density estimate (using the proportion of the parking lot space covered with carcasses — roughly within the circumference of each tree canopy). We do not know the location of the other photos within the perimeter of the treated area. Therefore, we report 95% confidence intervals (CI) from our calculations, representing the best estimate available of the number of carcasses that were on the ground on 17 June 2013. Using the eight photos, we estimate that the density of bumble bee carcasses was between 22.9 and 53.7 bees/m$^2$ (95% CI) leading to an estimate of between 45,830 and 107,470 total dead bumble bees. We acknowledge that this estimate comes from a few photos, under select trees, and are aware that this estimate is imprecise, though they are the best data available.

Colony Number Estimates and Predictions

The 492 sampled *B. vosnesenskii* represented 289 unique full-sibling families (i.e., one maternal queen, singly mated). Of the 289 families, the number of bees belonging to each family varied from one to four individuals, with 113, 153, 19, and 4 families of one, two, three, or four full siblings represented, respectively. We did not detect any full-sibling families with five or more individuals. Assuming random mating, the Colony algorithm estimated an effective population size of $N_e = 394$ (95% CI = 337, 458). Theory predicts that increasing the number of individuals sampled will increase our detection of unique full-sibling families in a given area. However, the number of unique families is expected to asymptote despite an increase in sample size. We used an asymptotic regression model to determine how many unsampled full-sibling families ($a$) are associated with our sampling of pesticide-killed bumble bees. All three parameters used to fit the model were significant ($P < 0.01$, $S_{sta}$ (residual standard error) = 3.72, $df = 24$). Based on our data, the horizontal asymptote ($a$), i.e., the estimated number of colonies is 596.30 ± 41.08 (SE) ($t = 14.52$, $P = 2.21e-13$, 95% CI = 511.52, 681.07) (Fig. 4). The parameters $b$ and $c$ used to fit the non-linear regression model are also significant ($b = 3.75 ± 1.41$, $t = 2.66$, $P = 0.01$, 95% CI = 0.84, 6.67; $c = −6.62 ± 0.09$, $t = −71.87$, $P < 2e-16$, 95% CI = −6.82, −6.44). Finally, applying the genotype data to ECM with Capwire resulted in a maximum likelihood estimate of 416 (95% CI = 382, 455) total colonies. Thus, we were able to detect 69.47% (289 detected colonies/416 MLE total colonies) in our sample of 492 genotyped individuals. Microsatellite data, colony output, and R scripts are available on GitHub (Koch 2021).

Discussion

Over the last several years, there have been studies documenting the effects of neonicotinoid insecticides on bee populations (van Lexmond et al. 2014, Pisa et al. 2015, Czerwinski and Sadd 2017, Woodcock et al. 2017) as well as an increased attention to the nectar chemistry of *Tilia* spp. and their role in mass bee deaths (Pawlikowski 2010, Koch and Stevenson 2017, Lande et al. 2019). Therefore, we cannot discount the possibility that the nectar chemistry of *Tilia* may have played some role in the Wilsonville bee fatalities in 2013. However, we present evidence from our pesticide analysis of *T. cordata* and *B. vosnesenskii* tissues that the Wilsonville incident is a case of non-target pesticide poisoning of a wild beneficial insect population. Though concentrations of dinofuran ranged from 800 to 3,767% of the LC$_{50}$ for *A. mellifera*, it is important to note that *A. mellifera* appear to have a higher threshold than bumble bees for acute lethal and sublethal effects of nitroguanidine neonicotinoids (Hopwood et al. 2016). Laboratory studies to determine the LC$_{50}$ of dinofuran on *Bombus* spp. would vastly improve our understanding of lethal and sublethal effects in this and other scenarios.

As a result of this single insecticide application, our study estimates that between 45,830 and 107,470 bumble bee fatalities occurred, mostly of one species, *B. vosnesenskii*. Using three independent approaches to estimating colony abundance (i.e., Colony, asymptotic analysis, and Capwire) with genotyped *B. vosnesenskii* workers, we estimate that at least 289 to 596 colonies in the Wilsonville area were affected. Furthermore, based on both those colony number and abundance calculations, we estimate that between 100 and 200 *B. vosnesenskii* colonies would have been killed to produce an abundance of this magnitude. *Bombus vosnesenskii* nests can be quite large, with average colony sizes reaching more than 1,000 individuals at the peak of the life cycle (Macfarlane et al. 1994). In our asymptotic analysis, we likely did not capture the number of colonies given our 95% confidence interval (511–681 colonies) due to a limited number of bees available for genetic analysis in our study (Fig. 4). Finally, based on museum data of *B. vosnesenskii* in Tuolumne County, CA, it is likely that the Wilsonville colonies are just beginning to peak in colony size, and are only beginning to produce sexuals, primarily fall gynes (Koch et al. 2012).

Our estimate in the number of colonies affected by insecticide application is likely conservative as we only sampled a small portion (*n* = 492) of the estimated number of bumble bee individuals using photographs and GIS analysis. Our analysis is unable to capture all the unsampled colonies, evident by the 113 individuals genotyped in which we did not detect a sibling in our pool of 492 individuals.
Fig. 4. Predicted colony numbers based on a three-parameter asymptotic regression model. Points represent the relationship between the number of genotyped individuals and the predicted number of full sibling colonies detected. Open circles represent observed numbers of colonies detected using a maximum likelihood detection algorithm on subsamples of the actual data in Colony (Jones and Wang 2010). The asymptotic regression model extends beyond the observed data to predict the number of full sibling colonies that will be detected with an increased sampling of individuals. Dashed lines represent the upper and lower 95% confidence interval of the prediction (α = 0.05).

A study by Mola et al. (2020) estimated 413 total colonies from 332 genotyped individuals. In their study, they estimated that they detected 46.49% of the colonies in the environment, whereas we detected 69.47% of the colonies in the environment. However, we also achieved a larger number of genotyped B. vosnesenskii individuals (n = 492) compared to Mola et al. (2020). Thus, increasing the sample size of genotyped workers will increase the number of detected colonies in an environment. Given these results, we hypothesize that increasing the sample size of genotyped individuals would have likely resulted in the discovery of more unique colonies impacted by insecticide poisoning event.

At the time of application (June 2013) the label for the applied dinotefuran product stated: ‘This product is highly toxic to bees exposed to direct treatment or residues on blooming crops or weeds. Do not apply this product or allow it to drift to blooming crops or weeds if bees are visiting the treatment area’. Because the label clearly stated the risk to bees if applied to blooming plants, the pesticide applicators were found to have violated ORS 634.372(4) (ORS 2019), which states that: ‘A person may not … Perform pesticide application activities in a faulty, careless or negligent manner’. The state determined that ‘Applying an insecticide, labeled as residually bee-toxic, to flowering trees in bloom on a warm day in late spring in a climatic area such as Wilsonville, Oregon, is a faulty, careless or negligent pesticide application activity in violation of ORS 634.372(4)’ (ODA 2014a). The violation resulted in the issuance of civil penalties by the ODA for violating ORS 634.372(4) (ORS 2019) that amounted to $1,665. In further response, the ODA took action to establish a permanent rule prohibiting the use of any product containing the neonicotinoid insecticides dinotefuran, clothianidin, imidacloprid, or thiamethoxam, regardless of application method, on linden trees, basswood trees, or other Tilia species. The permanent rule (OAR 603-057-0388; OAR 2015) went into effect on 27 February 2015.

Usage of neonicotinoid insecticides in the United States has vastly increased since they were first introduced in 1996 (Fig. 1) (Thelin and Stone 2013; Baker and Stone 2015; Baker 2015, 2016). The most recent estimates (including seed treatments) of pesticide application indicate that more than 3.5 million kg were applied across the USA in 2014 (Baker 2016). While there have been no direct links to broad scale pollinator declines, neonicotinoids have been implicated as plausible contributors to declines of many different species of bees at both lethal and sublethal levels (Whitehorn et al. 2012; Gill and Raine 2014; Pisa et al. 2015, 2017; Woodcock et al. 2016, 2017; Wood and Gouldson 2017).

In addition to the effects that were documented in this study, there were several other documented pesticide poisonings that took place in Oregon in 2013 and affected bumble bee populations (ODA 2014b). These poisonings include applications of either imidacloprid or dinotefuran that resulted in lethal and sublethal concentrations (0.020 – 0.069 ppm) of these chemicals in the flowers of treated Tilia trees, up to seven months after the initial application (ODA 2014b). All dead bumble bees that were sampled had significant levels of imidacloprid or dinotefuran. Thus, the effects of neonicotinoids from applications to ornamental trees on non-target insects like bumble bees are likely widespread in the United States. Since 2013, the Xerces Society has received several reports of dead bumble bees under treated trees throughout North America (Code 2019, pers comm).

Tilia cordata are common ornamental trees and have been advertised as beneficial to bees and other pollinators (Somme et al. 2016). However, T. cordata also attracts pest insects, such as aphids which produce a sticky substance called honeydew, which can drip onto parked cars and other surfaces below the trees and are thus targets of insecticide applications (Carter 1982). As such, the use of Tilia trees in urban environments, especially near parking lots where human encounters with honeydew are likely to draw attention, create a scenario where the application of an insecticide to lethally control aphids also impacts non-target insects such as bumble bees. It is likely that the Wilsonville pesticide incident is not an isolated case and occurs wherever Tilia cordata trees are planted in areas near human infrastructure. In response to the multiple incidents of pesticide poisoning of non-target insects, the State of Oregon has taken action to prevent this kind of event in the future by banning the use of all four nitroguanidine neonicotinoids on Tilia trees (OAR 2015). To our knowledge, the implications of this prohibition on human and bee health have not been assessed. In 2018, ODA produced a website (https://www.oregon.gov/oda/programs/Pesticides/Pages/PollinatorIssues.aspx) to help reduce the effects of pesticides on bees, including a document entitled ‘Alternatives to Neonicotinoid Insecticides (except for acetamiprid) for Use on Landscape Ornamentals’, which includes less-toxic alternatives like oils and soaps.

The focus of this study was to document the cause of the mass bumble bee mortality event that occurred in Wilsonville in 2013. However, our 2014 pesticide analysis of dinotefuran treated with soil drench methods raise the possibility of continued, and possibly sublethal effects of pesticide poisoning on pollinator populations. In 2014, the year after the initial application, ODA returned to the site to retest the treated trees. Two trees that received a foliar application in 2013 had an average concentration of dinotefuran in flowers of 9.2 ppm on 21 June 2013. However, dinotefuran was not detected in the 2014 pesticide analysis (ODA 2014b). Furthermore, two trees that received a soil drench application had an average concentration of dinotefuran in flowers of 0.066 ppm on 21 June 2013. While retesting the two T. cordata in 2014 (18 June) found an average concentration of 0.05 ppm, no bumble bee fatalities were observed (ODA 2014b). The fact that some trees had
higher concentrations in 2014 vs. 2013 implicates the possibility of a delayed mode of action in a soil drench application. The concentrations measured in 2014 are below the documented LC50 for dinotefuran (0.292–0.885 ppm), but above the level at which sublethal effects would likely be seen (0.0002 ppm–0.01 ppm; Fischer et al. 2014, Mommert et al. 2010, Elston et al. 2013). The sublethal effects of dinotefuran on bees have not been well studied, but sublethal effects (both acute and chronic) of the other three nitroguanidine neonicotinoids are seen at less than 1/100th LC50 levels (Yang et al. 2012, Elston et al. 2013, Laycock and Cresswell 2013, Moffat et al. 2015). Preliminary observations suggest that trees that received a soil drench had much lower acute bumper bee fatalities in 2013 (R. Hartfield pers. obs.). However, the residue results from 2014 suggest that the long-term effects of soil drench treatments may be much more significant, yet difficult to quantify. Other research has shown that application of neonicotinoid insecticides on woody landscape plants at any time of year result in nectar residues that exceed concentrations shown to have negative effects on bees, even when label directions to protect pollinators are followed (Mach et al. 2018).

While most of the bumper bees that died in this mass killing were of a single, locally common species (B. vosnesenskii) that likely had the representation, resiliency, and redundancy (Shaffer and Stein 2000) to recover, there is no way of telling if colonies of rare or at-risk species of bumper bees (i.e., B. occidentalis Greene 1858 [Hymenoptera: Apidae]) were affected (we sampled less than 1% of all bumper bees killed). Given the scale and scope of this event, it is likely that if any colonies were nearby, that they may have been severely affected, potentially disrupting conservation and recovery efforts. Bombus occidentalis has undergone severe declines west of the Cascade – Sierra Crest (Graves et al. 2020), however, the most recent observation of this species west of the Cascade – Sierra Crest in Oregon (in 2017, after the documented event) was less than 20 miles from the site of this bumper bee poisoning (The Xerces Society et al. 2019). The lethal effects of pesticide poisoning on non-target beneficial insects continue to occur today, as exemplified by the Wilsonville case and is a contributor to pollinator decline. Furthermore, even sublethal effects of pesticides are likely interacting with other factors associated with bumper bee decline including habitat loss, climate change, and disease. Recent research suggests that there may be no safe time of year to apply systemic neonicotinoid insecticides to trees and shrubs to avoid sublethal/lethal effects on bees, even if label directions and bee precaution language are followed (Mach et al. 2018). Combined, these negative factors will likely continue to hinder efforts to recover and repopulate species identified to be at risk of population decline and extirpation.

Tilia trees provide an interesting nexus for pollinator conservation. They bloom in late spring, an important time of year for pollinators. They initiates pest control measures at times making the trees candidates for pest control. While the use of broad spectrum, systemic insecticides, such as nitroguanidine neonicotinoids, may seem time and cost effective for landscape management, the potential negative consequences are evident from this study. These negative consequences are preventable, especially since aphids do not present threats to mature trees. Oregon has implemented a rule preventing the use of nitroguanidine neonicotinoids on Tilia trees as a clear message on the critical importance of supporting the health and safety of pollinator populations. While this rule would not prevent the more natural conditions that have led to mass bee deaths under Tilia trees for decades, it has the capacity to limit future population level effects to bumper bees and other pollinators from highly toxic insecticide applications at no cost to a tree or human health.

### Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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