Environmental Toxicology and Chemistry—Volume 38, Number 1—pp. 71–79, 2019
Received: 3 June 2018 | Revised: 7 August 2018 | Accepted: 7 November 2018

Environmental Chemistry

Exposures of Children to Neonicotinoids in Pine Wilt Disease Control Areas

Yoshinori Ikenaka,[a,b,*] Yuichi Miyabara,[c] Takahiro Ichise,[a] Shouta Nakayama,[a] Collins Nimako,[a] Mayumi Ishizuka,[a] and Chiharu Tohyama[d,e]

[a]Laboratory of Toxicology, Department of Environmental Veterinary Science, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido, Japan
[b]Water Research Group, Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa
[c]Institute of Mountain Science, Interdisciplinary Cluster for Cutting Edge Research, Shinshu University, Nagano, Japan
[d]Health, Environment, Science, and Technology International Consulting, Nerima, Tokyo, Japan
[e]Faculty of Medicine, University of Tsukuba, Ibaraki, Japan

Abstract: Neonicotinoid insecticides that have been on the market since 1992 have been used globally including in Japan. Because they are sprayed over forests and agricultural areas, inadvertent toxicity in nontarget insects (especially honey bees) and humans is a matter of public concern. However, information on exposure levels and potential health impacts of neonicotinoids in children living around sprayed areas is scarce. Thus, we determined neonicotinoid exposure levels in children living in communities where thiacloprid was used to control pine wilt disease. A total of 46 children (23 males and 23 females) were recruited for the present study, and informed written consent was obtained from their guardians. Urine specimens were collected before, during, and after insecticide spraying events; and atmospheric particulate matter was also collected. Concentrations of thiacloprid and 6 other neonicotinoid compounds were determined in urine samples and in atmospheric particulate matter specimens using liquid chromatography-electrospray ionization-tandem mass spectrometry. In urine specimens, thiacloprid concentrations were <0.13 μg/L and were detectable in approximately 30% of all samples. Concentrations of the other neonicotinoids, *N*-dm-acetamiprid, thiamethoxam, dinotefuran, and clothianidin, were 18.7, 1.92, 72.3, and 6.02 μg/L, respectively. Estimated daily intakes of these neonicotinoids were then calculated from urinary levels; although the estimated daily intakes of the neonicotinoids were lower than current acceptable daily intake values, the children were found to be exposed to multiple neonicotinoids on a daily basis. *Environ Toxicol Chem* 2019;38:71–79.

Keywords: Insecticide; Hazard/risk assessment; Pesticide risk assessment; Pesticide; Neonicotinoid; Children; Estimated daily intake

INTRODUCTION

Agrochemicals, including insecticides and herbicides, have been used to protect agricultural plants and forests from various kinds of pests, with general understanding of the balance of costs and benefits in society. However, exposures to agrochemicals can induce acute and chronic poisoning in sensitive populations, such as fetuses and infants as well as chemically sensitive populations at risk. In children, pesticide exposures reportedly occur via multiple routes, including diet, drinking water, inhalation, and skin absorption (Chensheng et al. 2000). Moreover, children living with parents who work with pesticides or who live in the proximity of pesticide-treated farmlands are more susceptible to higher pesticide exposures than others living in the same communities (Chensheng et al. 2000).

Children may be highly susceptible to the toxic effects of pesticides, with potential developmental, dietary, and physiologic consequences (Roberts et al. 2012) that are exacerbated by their rapid growth rates and high energy demands (caloric and oxygen requirements). Compared with adults, children drink more water, eat more Food, and breathe more air relative to their body weights, likely facilitating the accumulation of high doses of pesticides in their bodies (National Research Council 1993). In addition, blood–brain barriers of fetuses and neonates are immature during brain development, allowing the passage and accumulation of various chemicals, including pesticides, into fetal

This article includes online-only Supplemental Data.
* Address correspondence to y_ikenaka@vetmed.hokudai.ac.jp
Published online 28 November 2018 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.4316

brains (Zheng 2001). This may also result in higher circulating levels and enhanced toxicity of pesticides in children (Weiss et al. 2004). Taken together, the high susceptibility of children to pesticide exposures and the potential impacts of these compounds on children's health are considered to be serious public and academic concerns (Council on Environmental Health 2012).

Various insecticides, including organochlorines, organophosphates, and pyrethroids, have been developed and widely used for pest management. However, insecticides that are less toxic to humans relative to targeted species are highly sought, and since their introduction in 1992, neonicotinoids have been increasingly marketed. In 2008, neonicotinoids comprised 24% of all marketed agrochemicals (total volume of €6.330 billion), mainly replacing organophosphates (13.6%) and carbamates (10.8%; Jeschke et al. 2011). Global neonicotinoid use has continued to increase, and neonicotinoids have been registered for use in more than 120 countries, with a total production volume of US$2.5 billion across the globe (Akash et al. 2016). Neonicotinoids were designed as specific agonists of insect nicotine-like receptors (nAChRs). However, recent studies have shown that neonicotinoids can bind to not only insect nAChRs but also mammalian nAChRs, with nonnegligible dissociation constants (Kimura-Kuroda et al. 2012). These receptors are of critical importance to human brain function, especially during the development (Kandel et al. 2012) of memory, cognition, and behavior (Chen et al. 2014).

In Japan, pine trees are considered symbolic of the beauty of the natural environment and are appreciated in mountain and coastal environments and in gardens of historic sites. However, since the beginning of the 20th century, pine trees have been threatened by pine wilt disease in Japan (Proença et al. 2017), and the nematode *Bursaphelenchus xylophilus* was identified as the main cause. Because these worms are transmitted by the beetle species *Monochamus alternatus* (Mamiya 1988), pine trees have been protected from pine wilt by spraying insecticides over large areas using helicopters or jet-spray machines, although the effectiveness of such spraying practices remains to be proven.

Inhabitants of communities in such spraying zones are seriously concerned by these practices, and complaints of health problems and symptoms that are related to pesticide toxicity have been widely recorded. In previous studies, neonicotinoids were detected in urine samples from Japanese women and children, and neonicotinoid concentrations in 3-yr-old children living in Aichi Prefecture of Japan ranged from the limit of detection to 370 μg/g of creatinine, with a geometric mean of 4.16 μg/g of creatinine (Ueyama et al. 2015; Osaka et al. 2016). However, despite the ubiquitous use of neonicotinoids in pine wilt control areas, exposures and health impacts of neonicotinoids in these areas have not been reported. Thus, to assess neonicotinoid exposure levels of children (3–6 yr old) living in Nagano Prefecture of Japan, we determined concentrations of thiacloprid and 6 other neonicotinoids in urine specimens and estimated daily exposure levels with regard to acceptable daily intake values.

MATERIALS AND METHODS

The present study was approved by the Ethics Screening Committee of Hokkaido University (no. 28-1), and written informed consent was granted by all primary guardians prior to inclusion of children in the study.

Study areas

Subjects were selected from communities in Nagano Prefecture, which is located in the central part of Honshu Island, Japan. This prefecture has numerous valleys, high mountains, and forests; and the Japanese red pine (*Pinus densiflora*) is a predominant plant species in the local forests, which are periodically sprayed with insecticides to control pine wilt disease.

Study subjects

Children of 3 to 6 yr of age were recruited by advertising in city papers and in a local newspaper, and a total of 46 (23 males and 23 females) subjects were included. Mean ages of male and female subjects were 4.8 (range, 3–6) and 4.9 (range, 3–6) yr, respectively. Early morning urine samples (before breakfast) were collected by guardians before (26 May 2016), during (23 June 2016), and after (21 July 2016) insecticide spraying events using predistributed paper cups and transferred into plastic 15-mL centrifuge tubes. Tubes were then placed in ziplock bags and stored in household freezers until transfer to the Department of Toxicology, Faculty of Veterinary Science, Hokkaido University, for analysis.

Air sample collection

Air samples were collected at 2 sites (A and B) in the proximity of the residences of the studied subjects and at a reference site in Suwa City, Nagano Prefecture, where neonicotinoids have never been used to control pine wilt disease. Air samples (particulate matter) were collected using low-volume air samplers equipped with quartz filters (2500QAT-UP 55 mm; Pall life Sciences). Air sampling was performed in affected areas from 19 to 26 May 2016, from 17 to 24 June 2016, and from 14 to 22 July 2016 and in the reference area on 30 May 2016 and 6 June 2016.

Analysis of neonicotinoid levels in urine specimens

Urine was thawed, stirred, and allowed to stand for 1 h. Neonicotinoids and their metabolites were then extracted and purified using solid-phase extraction methods. In these procedures, Presep RPP cartridges (60 mg; Wako Pure Chemical Industries) were conditioned with 2 mL of methanol and 2 mL of distilled water, and 1.0-mL aliquots of urine were then thoroughly mixed with 5 ng (50 μL of 100 ppb solution) of internal standards and loaded into the cartridge. ENVIcarb/PSA (500 mg/300 mg; Sigma-Aldrich) cartridges were then conditioned with 10 mL of acetone and connected in series with Presep RPP cartridges and eluted with 8 mL of dichloromethane: acetonitrile (2:8, v/v) solution. After concentrating and dry-solidifying using a centrifugal concentrator (CVE-200D with UT-2000; EYELA), extracts were reconstituted with 100 μL of 3% (v/v) methanol solution and transferred into vials for analysis.

The liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI/MS/MS) instrument (Shimadzu 20 A series with LCMS8040; Shimadzu) was equipped with an RSpak DE-213 (2 mm i.d. × 150 mm) column (Showa Denko) for sample analyses. For high performance liquid chromatography (HPLC), solvents A and B comprised 0.1% (v/v) formic acid and 10 mM acetic acid in water and 0.1% (v/v) formic acid and 10 mM acetic acid in methanol, respectively, and were applied with the following gradient: $t = 0$ to 2 min, 20% solvent B; $t = 11$ min, 95% solvent B; $t = 11$ to 13 min, 95% solvent B. The column oven temperature and flow rate were 45 °C and 0.4 mL/min, respectively. Multiple reaction monitoring for mass spectrometry was programed as described in Table 1, and 6 neonicotinoids and an acetamiprid metabolite, *N*-dm-acetamiprid, were detected in the range of 96 to 102%. Precision of analysis of all 7 neonicotinoids was confirmed by multiple analysis, with a relative standard deviation of 10% (Table 1). Analytes were quantitated using internal standard methods, and calibration curves were generated for each analyte by mixing compounds with blank urine specimens to final concentrations of 0.05, 0.1, 0.2, 0.5, 1.25, 2.5, 3.75, and 5 ng/mL. During the preparatory stage of the present study, internal standards for nitenpyram and *N*-dm-acetamiprid were not commercially available. Hence, nitenpyram was quantified using the dinotefuran internal standard, dinotefuran-d3. Our choice of dinotefuran-d3 for nitenpyram quantification was based on the similarity in the retention times of nitenpyram and dinotefuran, as well the similarities in physicochemical properties of both compounds. In the absence of an internal standard, *N*-dm-acetamiprid was also quantified using acetamiprid-d6. Undetectable neonicotinoid levels were confirmed in urine specimens from a volunteer who mainly eats organic Food, and these were then used as blank urine. Extraction and purification of each calibration point were performed using the method described, and linearity exceeded $r^2 = 0.9$ in all calibration curves. Limits of quantitation (LOQs) were calculated as the lowest points on standard curves (Table 1) with relative standard deviations of <15% ($n = 5$) and signal-to-noise ratios of 5:1.

Measurements of urinary creatinine concentrations

Urinary creatinine concentrations were determined using Urinary Creatinine 10-Plate Detection Kits (Arbor Assays) according to the manufacturer's instructions.

Air sample preparation and analysis

Particulate matter from study and reference areas were prepared as described (Takenouchi and Aoi 2016). Briefly, shredded filters were put into 10-mL aliquots of ethyl acetate: acetone (9:1, v/v) solutions and sonicated for 5 min. Extracts were dried under a gentle stream of nitrogen gas and then dissolved in 1 mL of distilled water:acetone (4:1, v/v) solution. Finally, extracts were filtered through 0.45-μm pore membrane filters (DISMIC-25cs; Advantech) and transferred into HPLC vials for LC-ESI/MS/MS analyses.

Calculation of estimated daily intake from urinary neonicotinoid concentrations

Estimated daily intake of neonicotinoids was calculated from urinary neonicotinoid and creatinine concentrations using the following formula:

$$\begin{aligned}\text{Estimated daily intake (micrograms per day)} = {}& \text{urine neonicotinoid concentration} \\ & \text{(micrograms per gram if creatinine)} \\ & \times 0.3\ \text{(grams of creatinine per day)} \\ & \times 1/r\ \text{(urinary excretion correction coefficient)} \end{aligned} \quad (1)$$

Excreted creatinine levels were assumed to be 0.3 g/d, as shown previously in children (Sakurabayashi and Kousaka 1999). Because kinetics parameters for thiacloprid have not been established in humans, these were adopted from rat experiments ($r = 0.05$) in which 1.8 to 5.9% of orally ingested thiacloprid was excreted through urine (Pfeil and Tasheva 2006). Kinetics

TABLE 1: Selected neonicotinoids and their metabolites

Target neonicotinoids	LOQ (ppb)	Recovery rate (%)	RSD (%)	MRM	Polarity for ESI
Acetamiprid	0.05	102	7	223.0 > 126.0	+
Clothianidin	0.1	92	4	249.0 > 132.1	+
Dinotefuran	0.1	100	8	203.0 > 129.1	+
Imidacloprid	0.2	100	5	256.0 > 209.1	+
Nitenpyram	0.1	98	5	271.0 > 126.1	+
Thiacloprid	0.05	100	5	252.9 > 126.1	+
Thiamethoxam	0.1	96	4	291.9 > 211.0	+
N-dm-acetamiprid	0.05	101	9	208.9 > 126.1	+
Internal standards					
Acetamiprid d6	—	—	4	229.0 > 126.0	+
Clothianidin d3	—	—	4	249.0 > 132.1	+
Dinotefuran d3	—	—	7	206.0 > 132.3	+
Imidacloprid d4	—	—	10	259.7 > 179.2	+
Thiacloprid d4	—	—	6	256.9 > 126.1	+
Thiamethoxam d4	—	—	4	295.7 > 215.1	+

ESI = electrospray ionization; LOQ = limit of quantification; MRM = multiple reaction monitoring; RSD = relative standard deviation.

TABLE 2: Urinary neonicotinoids (micrograms per liter) in children before the insecticide spraying on 26 May 2016

		Selected percentile				
Neonicotinoid	Frequency (%)	25th	50th	75th	95th	Max
Acetamiprid	9	<LOD	<LOD	<LOD	0.21	0.53
Clothianidin	41	<LOD	<LOD	0.50	1.32	3.60
Dinotefuran	43	<LOD	<LOD	0.55	10.25	15.60
Imidacloprid	13	<LOD	<LOD	<LOD	0.35	4.70
Nitenpyram	0	<LOD	<LOD	<LOD	<LOQ	<LOQ
Thiacloprid	28	<LOD	<LOD	0.06	0.06	0.10
Thiamethoxam	28	<LOD	<LOD	0.10	0.26	0.85
N-dm-acetamiprid	91	0.27	0.39	0.71	3.48	11.16
ΣNEO	98	0.59	1.34	2.58	12.38	16.06

LOD = limit of detection; LOQ = limit of quantification; Max = maximum; NEO = neonicotinoid.

parameters for acetamiprid, clothianidin, dinotefuran, and imidacloprid in humans are reportedly $r = 0.586$, 0.596, 0.899, and 0.133, respectively (Harada et al. 2016). In the absence of established correction coefficients for excretions of nitenpyram and thiamethoxam in humans, we applied $r = 0.8$ and 0.6, respectively, for these neonicotinoids (Joint FAO/WHO Meeting on Pesticide Residues 2010; Food Safety Committee 2016). Finally, daily exposures to thiacloprid and other neonicotinoids were calculated by applying these coefficients to Equation 1. According to a previously reported kinetic study (Harada et al. 2016), most of acetamiprid, once absorbed into the body, is rapidly metabolized and excreted to urine as *N*-dm-acetamiprid. In the estimation of estimated daily intake values for acetamiprid, we incorporated *N*-dm-acetamiprid data into acetamiprid data.

Atmospheric contributions to estimated daily intakes of neonicotinoids in children from study areas were calculated using Equation 2, with the assumption that the daily breathing volume of 1- to 12-yr-old children is 8.7 m^3 (Kawahara et al. 2010):

$$\begin{aligned}&\text{Neonicotinoid intake from the atmosphere}\\&\text{(nanograms per day)} =\\&\text{atmospheric neonicotinoid concentration}\\&\text{(picograms per cubic meter)} \times \text{daily breathing volume}\\&\text{of children } (8.7\text{m}^3/\text{d})\end{aligned} \quad (2)$$

Statistical analysis

Statistical analyses were performed using JMP 12 (SAS Institute). Nonparametric Steel-Dwass tests were performed to compare seasonal variations in urinary neonicotinoid concentrations. Differences among groups were considered significant when $p < 0.05$ in all analyses.

RESULTS AND DISCUSSION

Neonicotinoids in children's urine

Concentrations, detection frequencies, and percentiles of urinary neonicotinoids in children living around target areas of thiacloprid (main ingredient of EcoOne-3 Flowable insecticide) spraying are summarized in Tables 2–4 were calculated before (May), during (June), and after (July) insecticide spraying was conducted. Frequencies of thiacloprid contents more than LOQ in early morning urine samples were 28, 30, and 33% before, during, and after insecticide spraying, respectively, and did not differ between sampling months (Tables 2–4; Steel-Dwass test, $p > 0.05$). Moreover, no significant changes were observed in plots of urinary thiacloprid concentrations against sampling times relative to insecticide spraying events (Figure 1). These analyses suggest that spraying of EcoOne-3-Flowable insecticide in target areas of the pine wilt disease prevention program did not cause excessive thiacloprid exposures in children. Alternatively, the days between insecticide spraying and urine sample collection may have been sufficient for clearance of

TABLE 3: Urinary neonicotinoids (micrograms per liter) in children during the insecticide spraying exercise (23 June 2016)

		Selected percentile				
Neonicotinoid	Frequency (%)	25th	50th	75th	95th	Max
Acetamiprid	11	<LOQ	<LOQ	<LOQ	0.27	0.54
Clothianidin	52	<LOQ	0.14	0.59	3.18	4.58
Dinotefuran	54	<LOQ	0.14	0.68	5.13	72.31
Imidacloprid	15	<LOQ	<LOQ	<LOQ	0.36	0.64
Nitenpyram	30	<LOQ	<LOQ	0.20	2.23	10.83
Thiacloprid	30	<LOQ	<LOQ	0.06	0.06	0.13
Thiamethoxam	37	<LOQ	<LOQ	0.12	0.67	1.71
N-dm-Acetamiprid	93	0.25	0.34	0.66	5.09	7.17
ΣNEO	100	0.59	1.79	4.13	10.85	75.09

LOQ = limit of quantification; Max = maximum; NEO = neonicotinoid.

TABLE 4: Urinary neonicotinoids (micrograms per liter) in children after the insecticide spraying exercise (21 July 2016)

		Selected percentile				
Neonicotinoid	Frequency (%)	25th	50th	75th	95th	Max
Acetamiprid	11	<LOQ	<LOQ	<LOQ	0.23	1.34
Clothianidin	49	<LOQ	<LOQ	0.64	3.24	6.02
Dinotefuran	49	<LOQ	<LOQ	0.21	1.54	8.58
Imidacloprid	18	<LOQ	<LOQ	<LOQ	0.39	1.48
Nitenpyram	27	<LOQ	<LOQ	0.19	0.26	0.63
Thiacloprid	33	<LOQ	<LOQ	0.06	0.06	0.10
Thiamethoxam	47	<LOQ	<LOQ	0.13	0.47	1.92
N-dm-acetamiprid	87	0.24	0.46	0.98	6.42	18.72
ΣNEO	100	0.59	1.14	2.68	11.79	19.33

LOQ = limit of quantification; Max = maximum; NEO = neonicotinoid.

neonicotinoids. Accordingly, a previous study showed that the first and terminal elimination-phase half-lives of thiacloprid were 2.2 and 19.0 h in male rats and 3.3 and 44.5 h in female rats, respectively (Pfeil and Tasheva 2006). EcoOne-3-Flowable insecticide was sprayed in the study area on 1 to 3, 9, 22, and 29 June; and urine samples were collected on 12 June 2016, only 1 d after spraying. However, urinary thiacloprid concentrations did not differ significantly before and after spraying, further suggesting that thiacloprid is rapidly metabolized and eliminated. These observations indicate that exposures to thiacloprid through EcoOne-3-Flowable insecticide spraying exercises are not commensurate with absorbed levels in children.

In a similar recent study, Osaka and associates (2016) analyzed neonicotinoid levels in urine samples from 3-yr-old children (108 boys and 115 girls) living in Aichi Prefecture, Japan, but did not detect thiacloprid in any of their samples. These discrepancies with the present data may reflect their reported LOQ for thiacloprid (0.32 ng/mL), which was much higher than our present LOQ of 0.05 ng/mL. Alternatively, Ueyama et al. (2015) observed a steady increase in urinary thiacloprid detection frequencies in specimens that were collected from adult women from 2003 to 2011, suggesting that the present data reflect an increasing trend of neonicotinoid use in Japan.

In addition to thiacloprid, 6 other neonicotinoid compounds that are not constituents of EcoOne-3-Flowable insecticides were detected in the children of the present study (Tables 2–4). Although acetamiprid was detected in only 8.5 to 12.8% of subjects, its major metabolite, *N*-dm-acetamiprid, was present in 86.6 to 93.5% of urine specimens. Detection frequencies of the other neonicotinoids were 28.3 to 46.6% for thiamethoxam, 43.4 to 54.3% for dinotefuran, and 41.3 to 52.2% for clothianidin. These detection frequencies in children were far higher than those of thiacloprid (Tables 2–4), and absolute urinary concentrations of these neonicotinoids were higher than those of thiacloprid (Table 2–4 and Figure 2).

Urinary concentrations of nitenpyram increased from May (below LOQ) to June (10.3 μg/L; Tables 2 and 3 and Figure 1), suggesting that nitenpyram exposures follow the consumption

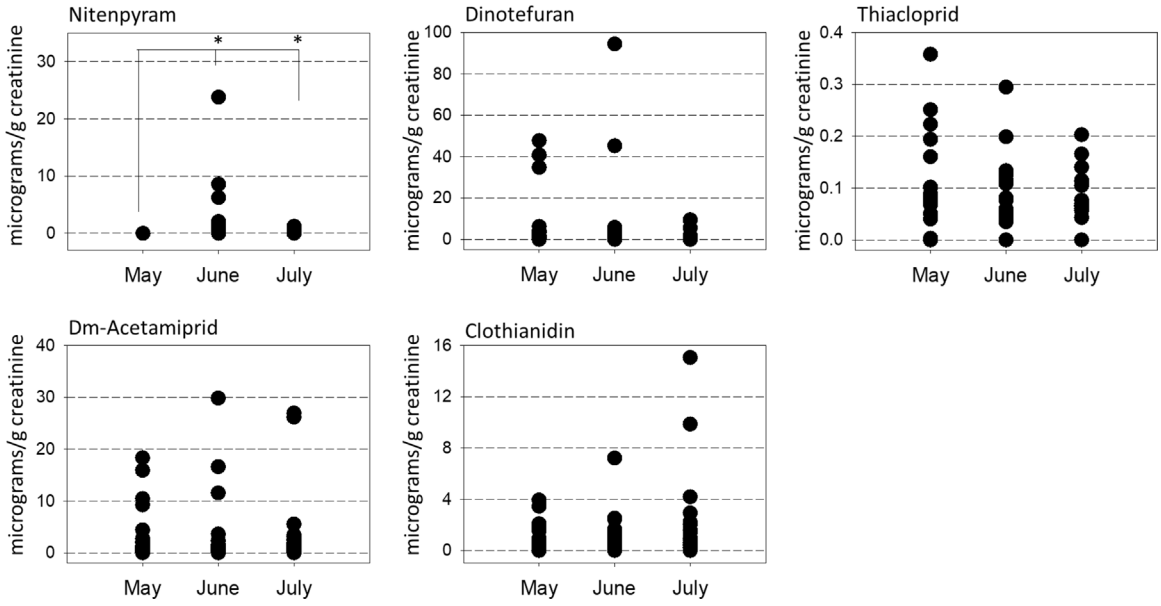

FIGURE 1: Time course of thiacloprid and other neonicotinoid concentrations in urine. Time course plots did not include urinary neonicotinoid concentrations below the limits of quantification.

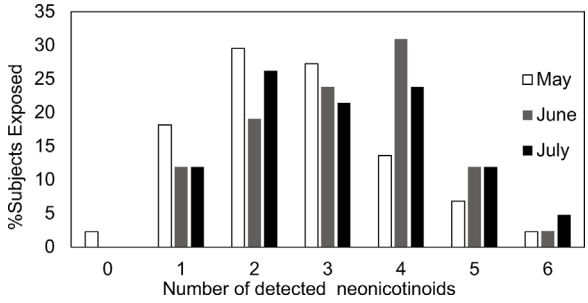

FIGURE 2: Multiple exposure evaluation of neonicotinoids among the children; most children were found to be exposed to multiple neonicotinoid compounds.

of agricultural products. Although pesticide use in rice, fruit, and vegetables from the study area usually increase during June, the precise contributions of domestic and international agricultural products to nitenpyram exposures in children remain unknown. In contrast, urinary concentrations of dinotefuran, thiacloprid, *N*-dm-acetamiprid, and clothianidin did not differ significantly during sampling periods (Tables 2–4 and Figure 1), indicating that exposures of the children to neonicotinoids occur at the present study areas irrespective of the timing of spraying of thiacloprid. Hence, further studies are needed to identify exact exposure sources of neonicotinoids in the present study area.

Children seemed to be exposed to multiple kinds of neonicotinoids in June and July compared to May, the observation of which may reflect a more active pest control activity in summer in this area. Generally, the majority (>80%) of the children were found to be exposed to multiple kinds of neonicotinoids in the study areas (Figure 2), warranting investigations of synergistic effects of neonicotinoids in these children.

Atmospheric neonicotinoids

Neonicotinoid concentrations in atmospheric particulate matter from sites A and B are presented in Table 5. Among these, atmospheric thiacloprid concentrations were 67.9, 25.8, and 35 pg/m^3 before, during, and after spraying of EcoOne-3-Flowable insecticide (Table 5), respectively. The comparatively high levels before spraying preclude associations with EcoOne-3-Flowable insecticide spraying activities in the present study, although our air sampling was performed 14 d after spraying of the EcoOne-3-Flowable insecticide, allowing residual thiacloprid to diffuse away before the sampling period. In contrast, atmospheric concentrations of thiacloprid in site B were 90 pg/m^3 during the spraying exercise, only 32 pg/m^3 before spraying, and 45 pg/m^3 after spraying. Hence, the elevated levels of thiacloprid observed in June likely follow variations in atmospheric thiacloprid concentrations. Similarly, Takenouchi and Aoi (2016) collected air samples during a similar spraying period at the Togura region in Chikuma City of Nagano Prefecture in 2013. Their determination of thiacloprid concentrations in atmospheric particulate matter showed increased thiacloprid concentrations from below LOQ (<35 pg/m^3) to 1900 pg/m^3 immediately after spraying exercises and restoration of baseline thiacloprid levels below the LOQ after only 1 d. These observations confirm that thiacloprid has a short atmospheric residence time, presumably because of its low vapor pressure (Supplemental Data, Table S2). The disparity between the trends of atmospheric thiacloprid concentrations observed in the report by Takenouchi and Aoi (2016) and that of the present study may be attributable to differences in sampling spots. Whereas Takenouchi and Aoi (2016) collected atmospheric particulate matter beside the sprayed spots, samples in the present study were collected in the residential area a few kilometers from the sprayed spots. Apparently, a greater proportion of the thiacloprid concentrations in the atmosphere might have either drifted away or settled on the ground at the time of sampling for the present study.

Estimated daily intake of neonicotinoids

The estimated daily intake of thiacloprid in children from the study areas was maximal at 2.15 μg/d, and 75th percentile amounts of intake were 0.287, 0.310, and 0.367 μg/d in May, June, and July, respectively (Tables 6–8). These amounts are within the acceptable daily intake of 12 μg/kg/d, which is equivalent to 180 μg/15 kg/d in children (Supplemental Data, Table S1).

In addition, we compiled estimated daily intake values of other neonicotinoids besides thiacloprid in children during the EcoOne-3-Flowable insecticide spraying exercise in June (Table 7). At an estimated daily intake of 15.2 μg/kg/d, daily intake of acetamiprid was about 1.4% of its acceptable daily

TABLE 5: Atmospheric neonicotinoid concentrations (picograms per cubic meter) in dust from sites A and B and the control site (EcoOne-3-Flowable nonspraying area)

	Site A			Site B			Control site
Neonicotinoid	19–26 May	17–24 June	14–22 July	19–26 May	17–24 June	14–22 July	30 May–6 June
Acetamiprid	54.2	77.5	35.5	58.3	60.6	47.5	43.6
Clothianidin	26.1	<LOQ	<LOQ	50.2	<LOQ	5.1	<LOQ
Dinotefuran	<LOQ	<LOQ	128.9	76.5	71.3	216.7	<LOQ
Imidacloprid	98.8	76.0	56.1	143.1	100.5	52.5	64.4
Nitenpyram	<LOQ	<LOQ	<LOQ	0.2	1.6	<LOQ	<LOQ
Thiacloprid	67.9	25.8	35.0	32.1	90.0	45.3	18.9
Thiamethoxam	64.2	54.2	44.7	<LOQ	<LOQ	44.2	<LOQ
N-dm-acetamiprid	7.9	7.0	6.1	8.3	5.8	6.3	6.8

LOQ = limit of quantification.

TABLE 6: Estimated daily intake of neonicotinoids in children before insecticide spraying exercise (26 May 2016)[a]

	EDI percentile (μg/day)					
Neonicotinoid	25th	50th	75th	95th	Max	%ADI
Acetamiprid	0.15	0.39	0.57	5.37	9.35	0.9
Clothianidin	—	—	0.30	1.02	1.99	0.1
Dinotefuran	—	—	0.23	9.22	15.90	0.5
Imidacloprid	—	—	—	1.05	11.0	1.3
Nitenpyram	—	—	—	—	—	—
Thiacloprid	—	—	0.29	1.29	2.15	1.2
Thiamethoxam	—	—	0.03	0.20	0.41	0.2
ΣNEO	0.62	1.02	2.40	12.5	19.1	0.1

[a]Values below limits of quantification are indicated by —. Estimated exposures were calculated assuming that creatinine excretion in children is 0.3 g/d. The excretion coefficients of acetamiprid, clothianidin, imidacloprid, and dinotefuran were retrieved from Harada et al. (2016) (r = 0.586, 0.596, 0.133, and 0.899, respectively). Excretion coefficients of nitenpyram, thiacloprid, and thiamethoxam were inferred from animal experiments (0.8, 0.05, and 0.6 for nitenpyram, thiacloprid, and thiamethoxam, respectively).
%ADI = percent of acceptable daily intake; EDI = estimated daily intake; Max = maximum EDI of neonicotinoids among subjects; NEO = neonicotinoid.

intake, whereas dinotefuran (31.4 μg/kg/d) was consumed at 1.0% of its acceptable daily intake, nitenpyram (8.92 μg/kg/d) was consumed at 0.1% of its acceptable daily intake, and thiamethoxam (0.813 μg/kg/d) was consumed at 0.3% of its acceptable daily intake (Table 7). Although these estimated daily intakes are relatively low compared to acceptable daily intakes, the maximal estimated daily intake of 51.6 μg/kg/d during the pesticide spraying season suggests that the EcoOne-3-Flowable spraying coincided with other agricultural activities that increased exposures of children to these neonicotinoids. The maximum estimated daily intake of imidacloprid in children was 11.1 μg/d (1.3% of its acceptable daily intake) before the EcoOne-3-Flowable insecticide spraying exercise (Table 6) but 1.23 μg/kg/d in June (0.1% of acceptable daily intake; Table 7) and 3.60 μg/kg/d (0.4% of acceptable daily intake; Table 8) in July, indicating limited effects of the spraying on imidacloprid exposure levels among the study subjects. Moreover, whereas peak estimated daily intakes for acetamiprid and imidacloprid were greater than those of all the other detected neonicotinoids, these did not exceed 2% of acceptable daily intake (Tables 6–8).

TABLE 7: Estimated daily intake of neonicotinoids in children during insecticide spraying exercise (23 June 2016)

	EDI percentile (μg/day)					
Neonicotinoid	25th	50th	75th	95th	Max	%ADI
Acetamiprid	0.41	0.24	0.521	5.17	15.20	1.4
Clothianidin	—	0.09	0.32	1.25	3.64	0.2
Dinotefuran	—	0.03	0.24	1.80	31.40	1.0
Imidacloprid	—	—	—	0.96	1.23	0.1
Nitenpyram	—	—	0.09	1.95	8.92	0.1
Thiacloprid	—	—	0.31	0.801	1.77	1.0
Thiamethoxam	—	—	0.07	0.41	0.81	0.3
ΣNEO	0.49	1.26	3.08	10.30	51.60	0.3

%ADI = percent of acceptable daily intake; EDI = estimated daily intake; Max = maximum EDI of neonicotinoids among subjects; NEO = neonicotinoid.

TABLE 8: Estimated daily intake of neonicotinoids in children after insecticide spraying exercise (21 July 2016)

	EDI percentile (μg/day)					
Neonicotinoid	25th	50th	75th	95th	Max	%ADI
Acetamiprid	0.15	0.31	0.90	3.05	13.7	1.3
Clothianidin	—	—	0.42	1.98	7.59	0.5
Dinotefuran	—	—	0.14	0.61	3.16	0.1
Imidacloprid	—	—	—	1.87	3.60	0.4
Nitenpyram	—	—	0.06	0.21	0.47	0.0
Thiacloprid	—	—	0.37	0.81	1.22	0.7
Thiamethoxam	—	—	0.06	0.38	1.01	0.4
ΣNEO	0.57	1.13	2.97	8.29	19.4	0.1

%ADI = percent of acceptable daily intake; EDI = estimated daily intake; Max = maximum EDI of neonicotinoids among subjects; NEO = neonicotinoid.

Finally, we determined the contributions of neonicotinoid inhalation from the atmosphere and showed that inhaled thiacloprid amounts were between 0.22 and 0.78 ng/d in the study area (sites A and B; Table 9), which are <1% of the estimated daily intake of thiacloprid (maximum 0.516 μg/d). We also found that inhalation from the atmosphere contributed very little to other neonicotinoid exposures (Table 9). Generally, neonicotinoids have very low vapor pressure (Raina-Fulton 2016; Supplemental Data, Table S2), meaning that most neonicotinoid compounds, especially imidacloprid and thiacloprid, have limited volatility and a short residence time in the atmosphere. Hence, it is possible that the thiacloprid which was used for the aerial spraying exercise in the present study area quickly settled on soil and/or water immediately after the spraying exercise, resulting in limited inhalation among the children. Collectively, the present data suggest that ingestion of neonicotinoids from foods and drinks contributes predominantly to total intakes by children and that inhaled neonicotinoid exposures are very limited in the present study areas.

Estimated daily intakes of all detected neonicotinoids in the children of the present study were far lower than acceptable daily intake values. However, a recent study indicates that

TABLE 9: Atmospheric neonicotinoid exposure estimates (nanograms per day) in children[a]

	Site A			Site B		
Neonicotinoid	May	June	July	May	June	July
Acetamiprid	0.47	0.67	0.31	0.51	0.53	0.41
Clothianidin	0.23	—	—	0.44	—	0.04
Dinotefuran	—	—	1.12	0.67	0.62	1.88
Imidacloprid	0.86	0.66	0.49	1.24	0.87	0.46
Nitenpyram	—	—	—	0.00	0.01	—
Thiacloprid	0.59	0.22	0.30	0.28	0.78	0.39
Thiamethoxam	0.56	0.47	0.39	—	—	0.38
N-dm-acetamiprid	0.07	0.06	0.05	0.07	0.05	0.05

[a]Neonicotinoid intake from the atmosphere (nanograms per day) = atmospheric neonicotinoid concentration (picograms per cubic meter) × child's daily breathing volume 8.7 m^3/d. The daily breathing volume in children of 1 to 12 years of age was assumed to be 8.7 m^3 (Koenig and Mar, 2000). Daily atmospheric exposures below limits of quantification are indicated by —.

exposures to no-obvious-adverse-effect levels (NOAELs) of neonicotinoids may induce adverse effects in animals. Specifically, whereas the NOAEL of clothianidin has been set at 9.7 mg/kg (Natural Resources Defense Council 2016), 5 mg/kg clothianidin reportedly induced anxiety-related behaviors in mice (Hirano et al. 2018), suggesting that the accepted NOAEL for clothianidin should be revised to a lower threshold. In the absence of robust evidence, a clothianidin NOAEL value of 0.5 mg/kg (one-tenth of 5 mg/kg) would correspond with an acceptable daily intake value of 0.005 mg/kg/d (using 100 as an uncertainty factor and 15 kg as the child's body weight). Under these conditions, the present clothianidin estimated daily intake of 7.6 μg/kg/d represents about 10% of the acceptable daily intake. In another study, Sun et al. (2016) reported that exposures to daily imidacloprid doses of 0.06 mg/kg promoted high-fat-induced adiposity and insulin resistance in male mice. These observations also imply that at 1% of the NOAEL (5.7 mg/kg), imidacloprid may affect energy metabolism via the 5′ adenosine monophosphate-activated protein kinase-α pathway. In the present study, the NOAEL for imidacloprid that we used was 0.006 mg/kg, which was one-tenth of the 0.06 mg/kg used in the Sun et al. study; therefore, on comparing the results, the estimated daily intake of imidacloprid in the present children was 2 times higher than the acceptable daily intake (currently 1.3% of the acceptable daily intake). Hence, further studies are warranted to precisely assess the toxicity of neonicotinoids in humans and adjust acceptable daily intake values accordingly.

CONCLUSIONS

In the present study, concentrations of the neonicotinoids acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid, and thiamethoxam were determined in urine samples from children living in areas where thiacloprid was used to control pine wilt disease. Subsequent analyses showed very limited neonicotinoid inhalation among children. However, the presence of 6 other neonicotinoids reflected high intakes of agricultural products by these children, although estimated intake levels of neonicotinoids were <2% of acceptable daily intake values. Finally, whereas current exposure levels of the compounds detected in the present study were far below the doses that induce acute toxicity, sufficient caution should be taken to avoid the potential cumulative impacts of these compounds in sensitive populations, especially among children and chemically sensitive individuals.

Acknowledgment—We express our sincere appreciation to the children and their parents who cooperatively participated in the present study. The recruiting of participants and collecting of urine specimens were conducted by M. Taguchi. The present study was supported in part by JSPS KAKENHI (JP16H0177906, to M.I.; 18H0413208, 15H0282507, 15K0055915, and 15K0055915, to Y. Ikenaka). The analyses were technically supported by K. Fujioka, M. Yagihashi, and N. Hirano. We also thank Enago (www.enago.jp) for the English language review.

Data Accessibility—Data, associated metadata, and calculation tools are available by contacting the corresponding author (y_ikenaka@vetmed.hokudai.ac.jp).

REFERENCES

Akash MS, Supowit SD, Halden RU. 2016. Mass balance assessment for six neonicotinoid insecticides during conventional wastewater and wetland treatment: Nationwide reconnaissance in United States wastewater. *Environ Sci Technol* 50:6199–6206.

Chen M, Tao L, McLean J, Lu C. 2014. Quantitative analysis of neonicotinoid insecticide residues in foods: Implication for dietary exposures. *J Agric Food Chem* 62:6082–6090.

Chensheng L, Fenske RA, Simcox NJ, Kalman D. 2000. Pesticide exposure of children in an agricultural community: Evidence of household proximity to farmland and take home exposure pathways. *Environ Res* 84:290–302.

Council on Environmental Health. 2012. Pesticide exposure in children. *Pediatrics* 130:1757–1763. Itasca, IL, USA. [cited 2018 February 15]. Available from: http://pediatrics.aappublications.org/content/130/6/e1757

Food Safety Committee. 2016. Pesticide evaluation report: Nitenpyram. Heisei Central Environmental Council, Japan. [cited 2018 June 20]. Available from: https://www.env.go.jp/council/10dojo/y104-63/ref02.pdf

Harada KH, Tanaka K, Sakamoto H, Imanaka M, Niisoe T, Hitomi T, Kobayashi H, Okuda H, Inoue S, Kusakawa K, Oshima M, Watanabe K, Yasojima M, Takasuga T, Koizumi A. 2016. Biological monitoring of human exposure to neonicotinoids using urine samples, and neonicotinoid excretion kinetics. *PLoS One* 11:1–16.

Hirano T, Yanaia S, Takada T, Yoneda N, Omotehara T, Kubota N, Minami K, Yamamoto A, Mantani Y, Yokoyama T, Kitagawa H, Hoshi N. 2018. NOAEL-dose of a neonicotinoid pesticide, clothianidin, acutely induce anxiety-related behavior with human-audible vocalizations in male mice in a novel environment. *Toxicol Lett* 282:57–63.

Jeschke P, Nauen R, Schindler M, Elbert A. 2011. Overview of the status and global strategy for neonicotinoids. *J Agric Food Chem* 59:2897–2908.

Joint FAO/WHO Meeting on Pesticide Residues. 2010. Pesticide residues in food, Report 2010. FAO plant production and protection paper 200:313-364. [cited 2018 June 13]. Available from: http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation10/2010_Evaluation.pdf

Kandel E, Schwartz J, Jessell T, Siegelbaum S, Hudspeth AJ. 2012. *Principles of Neural Science*, 5th ed. McGraw-Hill Education, New York, NY, USA.

Kawahara J, Tanaka C, Tanaka S. 2010. Estimation of minute ventilation rate of preschool children using tri-axial accelerometer. *J Japan Soc Atmo Env* 45:235–245.

Kimura-Kuroda J, Komuta Y, Kuroda Y, Hayashi M, Kawano H. 2012. Nicotine-like effects of the neonicotinoid insecticides acetamiprid and imidacloprid on cerebellar neurons from neonatal rats. *PLoS One* 7:1–11.

Koenig QI, Mar FT. 2000. Sulfur dioxide; Evaluation of current California air quality standards with respect to protection of children. Department of Environmental health, University of Washington. [cited 2018 June 13]. Available from: https://pdfs.semanticscholar.org/6b08/9b53ed322626f420b95e923e84c3807f6f10.pdf?_ga=2.205501648.1974096043.1543917218-1483343575154391721 8

Mamiya Y. 1988. History of pine wilt disease in Japan 1. *J Nematol* 20:219–226.

National Research Council. 1993. *Pesticides in the Diets of Infants and Children*. National Academies Press, Washington, DC. [cited 2018 March 28]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK236275/.

Natural Resources Defense Council. 2016. Clothianidin (CAS# 210880-92-5) GreenScreen® for safer chemicals (GreenScreen®) assessment. Bethesda, MD, USA. [cited 2018 March 28]. Available from: https://ntp.niehs.nih.gov/ntp/ohat/publiccomms/2016/attachment-3.pdf

Osaka A, Ueyama J, Kondo T, Nomura H, Sugiura Y, Saito I, Nakane K, Takaishi A, Ogi H, Wakusawa S, Ito Y, Kamijima M. 2016. Exposure characterization of three major insecticide lines in urine of young children in Japan—Neonicotinoids, organophosphates, and pyrethroids. *Environ Res* 147:89–96.

Pfeil R, Tasheva M. 2006. Thiacloprid. Thiacloprid X-X JMPR. [cited 2018 March 15]. Available from: apps.who.int/pesticide-residues-jmpr-database/Document/140

Proença DN, Grass G, Morais PV. 2017. Understanding pine wilt disease: Roles of the pine endophytic bacteria and of the bacteria carried by the disease-causing pinewood nematode. *Microbiologyopen* 6: e00145.

Raina-Fulton R. 2016. Neonicotinoid insecticides: Environmental occurrence in soil, water and atmospheric particles. Berlin, Germany. [cited 2018 June 10]. Available from: http://www.avidscience.com/wp-content/uploads/2016/08/PST-16-02.pdf

Robert JR, Karr CJ; Council on Environmental Health. 2012. Technical Report: Pesticide exposure in children. *Pediatrics* 130:e1765–e1788.

Sakurabayashi I, Kousaka I. 1999. *Inspection Dictionary*, 2nd ed. Otsuka Pharmaceutical, Otsuka, Tokushima, Japan.

Sun Q, Xiao X, Kim Y, Kim D, Yoon SK, Clark MJ, Park Y. 2016. Imidacloprid promotes high fat diet-induced adiposity and insulin resistance in male C57BL/6J mice. *J Agric Food Chem* 64:9293–9306.

Takenouchi T, Aoi T. 2016. Determination of drift of neonicotinoid insecticide thiacloprid caused by aerial spraying by liquid chromatography-tandem mass spectrometry. *J Environ Chem* 26:27–32.

Ueyama J, Harada HK, Koizumi A, Sugiura Y, Kondo T, Saito I, Kamijima M. 2015. Temporal level of urinary neonicotinoid and dialkylphosphate concentrations in Japanese women between 1994 and 2011. *Environ Sci Technol* 49:14522–14528.

Weiss B, Amler S, Amler RW. 2004. Pesticides. *Pediatrics* 113(Suppl. 3):1030–1036. [cited 2018 March 20]. Available from: pediatrics.aappublications.org/content/113/Supplement_3/1030

Zheng W. 2001. Neurotoxicology of the brain barrier: New implications. *Clin Toxicol* 30:711–719.

