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A nationwide survey of urinary concentrations of neonicotinoid insecticides in China

Tao Zhang[a,b,*], Shiming Song[a,b], Xueyuan Bai[a], Yuan He[a], Bo Zhang[a], Mingwei Gui[a], Kurunthachalam Kannan[c], Shaoyou Lu[d,**], Yanying Huang[a], Hongwen Sun[e]

[a] School of Environmental Science and Engineering, Sun Yat-Sen University, Guangzhou 510275, China
[b] Guangdong Provincial Key Laboratory of Environmental Pollution Control and Remediation Technology, Sun Yat-Sen University, Guangzhou 510275, China
[c] Wadsworth Center, New York State Department of Health, Department of Environmental Health Sciences, School of Public Health, State University of New York at Albany, Albany, NY 12201, USA
[d] School of Public Health (Shenzhen), Sun Yat-Sen University, Guangzhou 510006, China
[e] MOE Key Laboratory of Pollution Processes and Environmental Criteria, College of Environmental Science and Engineering, Nankai University, Tianjin 300071, China

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ABSTRACT

Neonicotinoid insecticides (NEOs) are emerging pesticides of concern due to their potential toxicity to non-target species (e.g., bees, fish and birds). China is an important producer and user of NEOs in the world. Studies on human exposure to NEOs in China are very limited. In this study, urinary levels of six NEOs, namely acetamiprid (ACE), clothianidin (CLO), dinotefuran (DIN), imidacloprid (IMI), thiacloprid (THD), and thiamethoxam (THM) were determined in 324 individuals from 13 cities in China. Across all sampling locations, total NEO concentrations (ΣNEOs; sum of six NEOs) were dominated by CLO (median: 0.24 ng/mL), IMI (0.21 ng/mL), THM (0.15 ng/mL) and DIN (0.14 ng/mL) collectively accounting for 98% of the concentrations. Urinary concentrations of each NEO varied depending on the sampling location with the median values ranged from 0.057 to 1.2 ng/mL for CLO, from 0.036 to 0.83 ng/mL for DIN, from 0.069 to 3.2 ng/mL for IMI, and from 0.062 to 0.45 ng/mL for THM. Sex-related differences in IMI, ACE and ΣNEOs concentrations were observed with males presenting significantly higher urinary levels than did females. All six NEOs were significantly positively correlated ($r = 0.135$ to 0.661, $p < 0.05$) with each other, suggesting that the exposure sources of NEOs are common or related. On the basis of urinary IMI levels, we calculated the median daily intake (DI; mean and range) of IMI to be 1.6 (4.1, < 0.02–55) μg/day, or 0.034 (0.11, < 0.0003–2.1) μg/kg bw/day. To our knowledge, this is the first study to document the ubiquitous occurrence of and human exposure to NEOs in China.

1. Introduction

Neonicotinoid insecticides (NEOs), including acetamiprid (ACE), clothianidin (CLO), thiacloprid (THD), imidacloprid (IMI), thiamethoxam (THM), dinotefuran (DIN) have become the most widely used class of insecticides in the world for their efficacy and systemic properties (Englert et al., 2017; Bass et al., 2015; Song et al., 2018; Zhang et al., 2019). It was estimated that NEOs accounted for > 25% of the global insecticide market in 2014 (Bass et al., 2015), and were replacements for organophosphorus (OPs), and pyrethroid (PYRs) insecticides (Anderson et al., 2015). NEOs are highly effective insecticides that do not permit development of resistance by pests and are relatively less toxic to mammals than other classes of insecticides (i.e. OPs, PYRs, and carbamates) (Anderson et al., 2015). However, concerns over potential health effects of NEOs are mounting, as exemplified by their toxicity to non-target organisms (e.g., honey bees and birds) (Rundlöf et al., 2015; Hallmann et al., 2014; Han et al., 2018; Cimino et al., 2017), and their potential to adversely effects of humans (Han et al., 2018).

Several studies reported harmful effects of NEOs on bees, and suggested a link between high concentration of NEO exposure and decline in the diversity and distribution of bees (Rundlöf et al., 2015; Balfour et al., 2017). Another study further reported an association between NEO exposure and decline in the populations of insectivorous birds (Hallmann et al., 2014). Several in vitro and in vivo studies indicated that NEOs can be toxic to mammals, and exert reproductive, hepatic,

* Correspondence to: T. Zhang, School of Environmental Science and Engineering, Sun Yat-Sen University, 135 Xingang West Street, Guangzhou 510275, China.
** Correspondence to: S. Lu, School of Public Health (Shenzhen), Sun Yat-Sen University, 132 Waihuan Dong Road, Guangzhou Higher Education Mega Center, Guangzhou 510006, China.
E-mail addresses: zhangt47@mail.sysu.edu.cn (T. Zhang), lushaoyou2005@163.com (S. Lu).

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neurological and genotoxic effects (Han et al., 2018; Cimino et al., 2017; Kimura-Kuroda et al., 2012; Gu et al., 2013; Kapoor et al., 2011; Toor et al., 2013). The European Food Safety Authority (EFSA) Panel on Plant Protection Products indicated that ACE and IMI may adversely affect the development of neurons and brain structures associated with the functions such as learning and memory (EFSA, 2013). Furthermore, an in vitro study revealed that NEOs are harmful to mammalian sperm function and embryonic development (Gu et al., 2013; Hirano et al., 2015), suggesting that NEOs are potential reproductive toxicants to humans. Therefore, assessment of human exposure to NEOs is necessary to evaluate potential health risks.

Several studies reported urinary concentrations of NEOs in populations from Japan, Sri Lanka, and Spain (Ueyama et al., 2014, 2015; Osaka et al., 2016; Kabata et al., 2016; López-García et al., 2017). Urinary concentrations of NEOs in Japanese populations steadily increased between 1994 and 2011, with IMI found at a detection rate (DR) of > 65% (Ueyama et al., 2015). The urinary NEOs concentrations in Sri Lanka ranged from 0.086 to 2.6 ng/mL (Kabata et al., 2016).

Furthermore, very few studies reported urinary NEOs concentrations in Chinese populations (Wang et al., 2015; Zhang et al., 2018). Wang et al. (2015) analyzed IMI in 295 urine samples and reported that DR of IMI were 100% and 95% in rural and urban areas, respectively. Zhang et al. (2018) measured 7 NEOs in a few urine samples (n = 10) from China. However, these studies were limited to a single location with a small number of participants. It is necessary to conduct nationwide survey of urinary NEOs in China, because China is an important producer and user of NEOs globally (Liang et al., 2017).

In this study, we determined urinary concentrations of 6 NEOs, namely ACE, CLO, DIN, IMI, THD and THM in general populations (n = 324) from 13 cities (12 provinces) of China. The influence of age and gender on urinary NEOs levels was examined, and domestic and global differences in concentrations and composition profiles of NEOs were explored. As a class of emerging pesticides, a survey of urinary NEOs concentrations covering a large geographical area provided information on the levels, profiles, spatial distributions, source contribution, and human health risk of NEOs in China.

2. Materials and methods

2.1. Chemical and reagents

The native standards of IMI, THM, ACE, CLO, and THD and their corresponding internal standards, IMI-d_4, THM-d_3, ACE-d_3, CLO-d_3, and THD-d_4 were purchased from Sigma-Aldrich (St. Louis, MO, USA) and all were > 97% purity. The native standard of DIN was also obtained from Sigma-Aldrich (> 97% purity; St. Louis, MO, USA); while IMI-d_4 was used as the internal standard of DIN due to that its internal standard was not available. Formic acid (purity > 95%) and β-glucuronidase were obtained from Sigma-Aldrich. Methanol (HPLC-grade), acetonitrile (HPLC-grade) and ethyl acetate (purity > 99%) were purchased from Merck (Damstadt, Germany). Milli-Q water was used in all experiments and was obtained through a Milli-Q system (Barnstead International, Dubuque, IA, USA).

2.2. Sampling locations and sample collection

Sample collection was approved by the Institutional Review Board of Sun Yat-sen University, Guangzhou, China. Before sampling collection, all donors (n = 324) provided informed consent and completed questionnaire surveys with information regarding age, gender and place of residence (Table S1). Urine samples (first morning voids) were collected from each investigated participant living in 13 cities (i.e., urban areas) of China during the winter of 2012 (n = 4 locations) or 2017 (n = 9 locations) (Fig. 1). The sampling locations were, Guilin in Guangxi Province (GL-Guangxi, n = 27), Chifeng in Inner Mongolia Province (CF-Inner Mongolia, n = 26), Maoming in Guangdong Province (MM-Guangdong, n = 27), Nantong in Jiangsu Province (NT-Jiangsu, n = 29), Dongying in Shangdong Province (DY-Shandong, n = 30), Datong in Shanxi Province (DT-Shanxi, n = 30), Lhasa and Shigates in Tibet Province (LS&SG-TB, n = 18), Yuxi in Yunnan Province (YX-Yunnan, n = 24), Nanchong in Sichuan Province (NC-Sichuan, n = 19), Nanchang in Jiangxi Province (NC-Jiangxi, n = 36), Yueyang in Hunan Province (YY-Hunan, n = 28), and Shijiazhuang in Hebei Province (SJZ-Hebei, n = 30). The 13 cities included in this study were distributed Northern (n = 4), Eastern (n = 3), Southern (n = 2), and Southwestern (n = 4) China. The donors comprised 195 males and 115 females (unknown gender: n = 14) with the ages ranging from 1 to 97 yrs. (< 18 yrs.: n = 111; 18–50 yrs.: n = 144; > 50 yrs.: n = 58; unknown: n = 11). All donors were healthy individuals with no known infectious diseases and occupational exposed. Approximately 50 mL urine samples were collected directly in polypropylene (PP) tubes, and the samples were stored cold (2–4 °C) or frozen until shipped on dry ice to Sun Yat-sen University. All of the samples were frozen at −20 °C until analysis.

2.3. Sample preparation

Urine samples were extracted for the analysis of NEOs based on a previous study (Song et al., 2019; Zhang et al., 2016). Briefly, 3 mL of urine sample was transferred into a 15 mL PP tube and spiked with 10 μL of internal standard mixture (0.5 ng/μL). The samples were mixed with 0.3 mL of 1.0 M ammonium acetate that contained 66 units of β-glucuronidase (prepared by spiking 150 μL of β-glucuronidase into 100 mL of 1.0 M ammonium acetate solution) to adjust the pH to 5.5 and then incubated at 37 °C for 12 h. Thereafter, the samples were extracted twice with 4 mL of ethyl acetate. For each successive extraction, the mixture was shaken vigorously for 60 min and then centrifuged at 3000 ×g for 5 min. The supernatants were combined and washed with 1.0 mL of Milli-Q water. After shaking vigorously for 10 min and centrifugation at 3000 ×g for 10 min, the supernatant was transferred into a 15 mL PP tube and concentrated to near-dryness using a gentle nitrogen stream. The residues were reconstituted in 0.5 mL of methanol and vortex mixed prior to instrumental analysis.

2.4. Instrumental analysis

Six NEOs were identified and quantified by isotope-dilution high performance liquid chromatography (HPLC, Shimadzu, Japan) coupled with a Q-Trap 5500 mass spectrometer (MS/MS, Applied Biosystems, Foster City, CA). Chemical separations were performed on an Atlantis®d-C18 column (5 μm, 2.1 × 150 mm, Waters), which was maintained at 40 °C. A 10 μL aliquot of the extract was injected and the mobile phase flow rate was set at 0.6 mL/min. The mobile phases were 0.1% formic acid in Milli-Q water (solvent A) and acetonitrile (solvent B). The gradient elution program was set as follows: 0–1.5 min, 60% solvent A; 1.5–4 min, 60%–50% solvent A; 4–7 min, 50%–0% solvent A, held for 1 min; 8–8.5 min, 0%–90% solvent A; and 8.5–11 min, linear gradient returned to the initial composition of 60% solvent A, held for 3 min. The total analytical time was 14 mins.

Electrospray ionization positive mode (ESI^+) was used for the identification of target compounds. The multiple reaction monitoring (MRM) mode was used for the quantification of NEOs with a dwell time of 50 ms. The source temperature was 450 °C, and the ionization voltage was 4500 V. The MRM transition monitored and other mass spectrometric parameters (i.e. precursor ion, product ion, declustering potential, entrance potential, collision energies, and collision cell potential) are shown in Table S2.

In this study, two injections have been conducted for chemical analysis: the first injection was for the determination of ACE, CLO, IMI, THD and THM, while the second injection was for DIN analysis only. Urinary DIN levels were not analyzed in LS&SG-Tibet and YX-Yunnan due to that urine samples were not available when conducting the

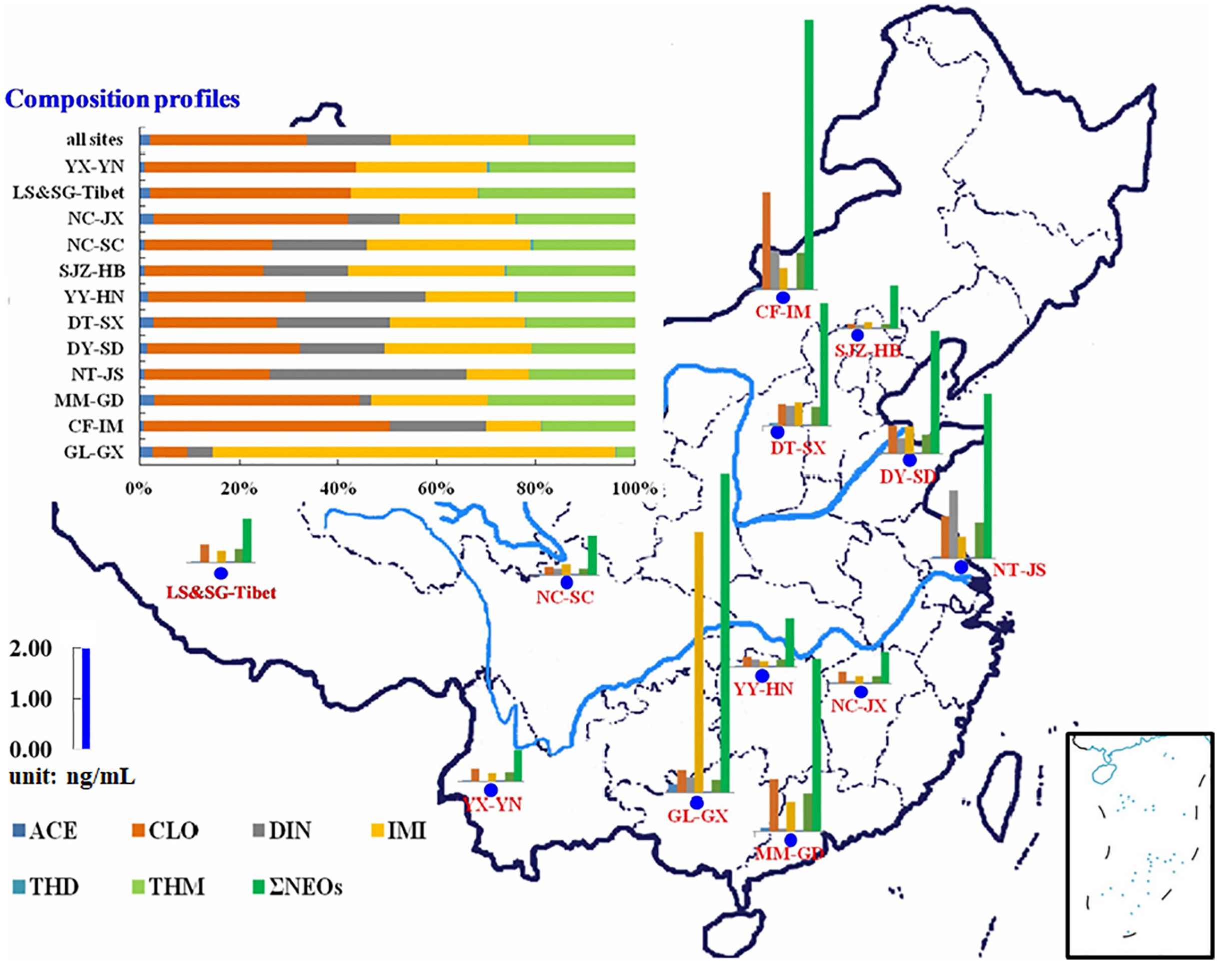

Fig. 1. The geographic distribution of urinary neonicotinoid concentrations and their composition profiles in different locations in China. *The sampling locations were: Shijiazhuang in Hebei Province (SJZ-HB), Yueyang in HuNan Province (YY-HN), Nanchang in JiangXi Province (NC-JX), Nanchong in Sichuan Province (NC-SC), Yuxi in YunNan Province (YX-YN), Shigatse and Lhasa in Tibet (LS&SG-Tibet), Datong in ShanXi Province (DT-SX), Dongying in ShanDong Province (DY-SD), Nantong in JiangSu Province (NT-JS), Maoming in GuangDong Province (MM-GD),Chifeng in Inner Mongolia* (CF-IM), *and Guilin in GuangXi Province (GL-GX). DIN was not analyzed in samples from LS&SG-Tibet and YX-YN due to these samples were not available; DIN was excluded from the estimation of ΣNEOs values in LS&SG-Tibet and YX-YN. Data on urinary NEOs in LS&SG-Tibet and YX-YN were also excluded from the calculation of NEOs composition profiles across all sites.*

second injection.

2.5. Quality assurance and quality control

Matrix-spike recoveries of individual NEOs through the analytical procedure were determined by spiking NEOs (5.0 ng in each) into randomly selected urine samples. The recoveries of NEOs ranged from 76% to 107% with relative standard deviations (RSDs) of < 7%. Five corresponding internal standards (i.e., IMI-d_4, THM-d_3, ACE-d_3, CLO-d_3, and THD-d_4) of target compounds were spiked into all urine samples (5.0 ng in each) prior to extraction, and the recoveries of internal standards spiked into samples ranged from 70% to 92%, with the RSDs of < 10%. One instrumental blank (i.e., methanol injection) and one procedural blank (i.e., Milli-Q water passed through entire procedure) were analyzed for every 20 samples to test for contamination arising from reagents and glassware. All the blanks were free of detectable concentrations of target NEOs.

A 12-point calibration curve was prepared over a concentration range of 0.002 to 20.0 ng/mL and this calibration standard was injected before every 30 samples. The regression coefficients (r^2) of calibration curve were > 0.99. The limits of quantification (LOQs) of NEOs in urine, defined as ten times the signal-to-noise (S/N) ratio, ranged from 0.0005 (THD) to 0.02 (DIN, IMI and THM) ng/mL; furthermore, the limits of detection (LOD) ranged from 0.0002 (THD) to 0.006 (DIN, IMI and THM) ng/mL.

2.6. Statistical analysis

Data analysis was performed with SPSS Version 19.0. The concentrations below the LOQ were assigned a value equal to half of the LOQ for the calculation of geometric mean (GM), median and arithmetic mean. The sum concentration of six NEOs is denoted as ΣNEOs. The concentrations of NEOs were tested for normality using Kolmogorov – Smirnov test. Differences between groups were analyzed by one-way ANOVA when the two sets of data were distributed normally; otherwise, Mann Whitney *U* test was used. The data set became normally distributed after log transformation. Therefore, Pearson correlation coefficients were used to analyze the relationship between different various demographic variables. The statistical significance was set at p-value ≤ 0.05.

3. Results and discussion

3.1. Urinary NEOs concentrations

Concentrations (GM, median, minimum and maximum) of individual NEOs and ΣNEOs in urine samples (n = 324) collected from 12 regions in China are presented in Table 1. All target NEOs, CLO (DR:

Table 1
Urinary concentrations (ng/mL) of neonicotinoid insecticides in people living in 12 different regions (13 cities) in China[a].

	ACE	CLO	DIN	IMI	THD	THM	ΣNEOs[h]
LOQ	0.0007	0.002	0.002	0.003	0.003	0.002	0.0007
All sites[e,f]							
DR[b] (%)	96	99	96	97	92	98	100
Median	0.014	0.24	0.14	0.21	0.0016	0.15	1.2
Min	< LOQ[d]	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.044
Max	0.61	17	18	7.3	0.18	8.4	20
GM[c]	0.012	0.24	0.13	0.19	0.0017	0.15	1.1
Mean	0.035	0.54	0.46	0.55	0.0034	0.41	2.1
By sampling sites (median values)							
GL-Guangxi	0.10	0.28	0.19	3.2	0.0053	0.15	3.9
CF-Inner Mongolia	0.02	1.2	0.47	0.27	0.002	0.45	3.3
MM-Guangdong	0.046	0.63	0.036	0.36	0.0014	0.45	2.1
NT-Jiangsu	0.019	0.52	0.83	0.26	0.0028	0.44	2.0
DY-Shandong	0.017	0.34	0.19	0.33	0.0009	0.23	1.5
DT-Shanxi	0.03	0.26	0.24	0.29	0.0015	0.23	1.5
YY-Hunan	0.0065	0.12	0.092	0.069	0.0014	0.09	0.59
SJZ-Hebei	0.0024	0.057	0.041	0.076	0.0009	0.062	0.53
NC-Sichuan	0.0038	0.10	0.075	0.13	0.0019	0.08	0.48
NC-Jiangxi	0.0098	0.14	0.037	0.084	0.0013	0.084	0.38
LS&SG-Tibet	0.011	0.22	NA[g]	0.14	0.0013	0.17	0.54
YX-Yunnan	0.0037	0.16	NA[g]	0.1	0.002	0.11	0.38

[a] Two significant digits have been used for concentration values, and 1.0 ng/mL = 1000 ng/L.
[b] DR = detection rates.
[c] GM = geometric mean values.
[d] < LOQ = concentrations value lower than LOQ.
[e] The estimated results (i.e., DR, median, minimum, maximum, GM and mean values) on urinary levels of ACE, CLO, IMI, THD and THM among all individuals were based on data from all 13 cities.
[f] The estimated results (i.e., DR, median, minimum, maximum, GM and mean values) on urinary levels of DIN among all individuals were based on data from 10 cities due to DIN was not analyzed in samples from LS&SG-Tibet and YX-Yunnan.
[g] NA = data are not available.
[h] ΣNEOs represent the sum urinary concentrations of all six compounds, and data from LS&SG-Tibet and YX-Yunnan were excluded from ΣNEOs estimation.

99%), THM (98%), IMI (97%), DIN (96%), ACE (96%), and THD (92%), were frequently detected in urine samples. CLO was found at the highest median concentration, 0.24 ng/mL (range: < LOQ to 17 ng/mL), followed by IMI at 0.21 ng/mL (< LOQ to 7.3 ng/mL), THM at 0.15 ng/mL (< LOQ to 8.4 ng/mL), and DIN at 0.14 ng/mL (< LOQ to 18 ng/mL); and the median concentrations of ACE and THD were 0.014 (< LOQ to 0.61 ng/mL) and 0.0016 ng/mL (< LOQ to 0.18 ng/mL), respectively (Table 1). Our results suggest that Chinese population is widely exposed to NEOs.

Few studies have reported urinary NEOs levels in China (Wang et al., 2015; Zhang et al., 2018). Wang et al. (2015) analyzed urinary IMI in donors from a village (n = 41; DR: 100%; median: 0.20 ng/mL) and a town (n = 20; DR: 95%; median: 0.15 ng/mL) in Shandong Province, China. Zhang et al. (2018) analyzed 9 NEOs in a 10 children urine samples living in Hangzhou, Zhejiang Province, China; and IMI (DR: 80%), THM (70%), ACE (80%) and CLO (70%) were detected at concentrations ranging from < LOQ - 2.7, < LOQ -1.4, < LOQ - 0.93, and < LOQ - 1.9 ng/mL, respectively.

Although the concentrations of NEOs in human urine were rarely reported in China, several studies have documented urinary OP and PYR insecticide levels in this country (Ding et al., 2012a, 2012b; Ding and Bao, 2014; Zhang et al., 2014; Wang et al., 2017; Chen et al., 2016; Ye et al., 2017). NEOs were developed to replace OPs and PYRs. A combination of all three classes of insecticides may be used in farms in recent years. We compared urinary levels of these three classes of insecticides to examine composition profiles of human exposure in China. The median urinary levels (blue column in Fig. S1) of NEOs were one to three orders of magnitude lower than those of urinary OPs metabolites (mOPs) (diethylphosphate, DEP: 1.2 to 7.1 ng/mL; diethylthiophosphate, DETP: 0.55 to 6.3 ng/mL; dimethylphosphate, DMP: 2.5 to 18 ng/mL; and dimethylthiophosphate, DMTP: 0.78 to 8.5 ng/mL) reported in China (Ding et al., 2012a; Ding and Bao, 2014; Zhang et al., 2014; Wang et al., 2017). Even the highest median urinary values of each NEO obtained among all sites (green column as shown in Fig. S1) were still lower than those of mOPs. In comparison with those of urinary PYR metabolites (mPYRs) concentrations (3-phenoxybenzoic acid, 3-PBA: 0.39 to 1.2 ng/mL; cis-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid, cisDCCA: 0.18 to 0.46 ng/mL; trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid, trans-DCCA: 0.57 to 1.2 ng/mL), median urinary levels of NEOs were slightly lower (Ding and Bao, 2014; Chen et al., 2016; Ye et al., 2017). However, the median urinary CLO concentrations from CF-Inner Mongolia (1.2 ng/mL), and urinary IMI concentrations from GL-Guangxi (3.2 ng/mL) were higher than those of urinary mPYRs reported in China (Ding and Bao, 2014; Chen et al., 2016; Ye et al., 2017). Overall, the profiles of three classes of insecticides in urine from China were in order of mOPs > > mPYRs ≈ NEOs, a pattern similar to that observed in Japan (Osaka et al., 2016).

It is worth to note that exposure to NEOs was from the measurement of parent compounds whereas metabolites were measured for OPs and PYRs. Nauen et al. (2003) reported that THM can be metabolized to CLO. Further, a study showed that approximately 85% of IMI was converted into unidentified metabolites in human body (Harada et al., 2016). Therefore, urinary concentrations of unidentified NEOs metabolites might be higher than those of their parent compounds and further studies are needed on this regard.

3.2. Spatial distribution of NEOs in China

Both concentrations (Table 1) and composition profiles (Fig. 1) of urinary NEOs varied among sampling locations in China. The highest median urinary concentration of ΣNEOs was found in GL-Guangxi, with a value of 3.9 ng/mL (range: 0.27–12 ng/mL), followed by those from CF-Inner Mongolia (median: 3.3 ng/mL), MM-Guangdong (2.1), NT-Jiangsu (2.0), DY-Shandong (1.5), and DT-Shanxi (1.5). Relatively low concentrations of ΣNEOs were found in LS&SG-Tibet (0.54), YX-Yunnan

(0.38), NC-Jiangxi (0.38), NC-Sichuan (0.48), YY-Hunan (0.59) and SJZ-Hebei (0.53), with the median values < 1.0 ng/mL. In general, higher urinary concentrations of NEOs were found in Southern (GL-Guangxi and MM-Guangdong), Eastern (NT-Jiangsu and DY-Shandong), and Northern (CF-Inner Mongolia) China, particularly in coastal provinces, than in Central (NC-Jiangxi and YY-Hunan) and Western (YX-Yunnan, NC-Sichuan and Tibet) China (Fig. 1). The region-specific variations in pesticides usage in China showed decreasing trends from Eastern to Western, or from Southern to Northern (Yang et al., 2013). The consumption rates of pesticides were estimated to be 651,000, 579,000 and 156,000 t in Eastern, Central, and Western China, respectively, in 2004 (Yang et al., 2013). Furthermore, Guangxi, Guangdong, and Shandong reported a consumption rate of pesticides (per hectare) higher than the national average (11 kg/ha) (Yang, 2015). The regional distribution of urinary ΣNEOs concentrations in China was generally consistent with the pattern of pesticide usage nationwide. However, NC-Jiangxi and YY-Hunan are located in Jiangxi and Hunan provinces, respectively, of Central China, where large amount of pesticides is consumed (> 11 kg/ha), but the urinary levels of NEOs were low in these locations. This may be attributed to small sample size, and further studies with larger sample size are needed to identify the relationships between pesticides usage and urinary NEOs levels.

The composition profiles of urinary NEOs levels for all sampling sites are shown in Fig. 1. In general, CLO was the most abundant NEO, accounting for 32% of ΣNEOs concentrations, followed, in decreasing order, by IMI 28%, THM 21%, DIN 17%, ACE 1.8%, and THD 0.2% (Fig. 1). Overall, CLO, IMI and THM collectively accounted for 98% of the ΣNEOs concentrations. It has been reported that CLO, IMI and THM are the most widely used NEOs in the world, accounting for 14%, 35% and 36%, respectively, of the NEOs market in 2011 (M. Zhang et al., 2012). The profiles found in urine reflect the usage pattern of NEOs. In addition, these three NEOs are mainly applied to agricultural crops such as fruits, vegetables and paddy (Casida, 2011; S. Zhang et al., 2012; Lu et al., 2018). Higher consumption of fruits and vegetables in China (Wu and Li, 2015), and the usage of NEOs on these agricultural crops support the composition profiles of urinary NEOs observed in this study.

The composition profiles of urinary NEOs in most sampling locations were similar with those of the profiles observed when all sampling sites were combined. Nevertheless, urine samples from GL-Guangxi showed a completely different profile in which IMI (82%, 3.2 ng/mL) was the predominant compound; and the contribution of DIN was high at 40% of ΣNEOs in NT-Jiangsu. Furthermore, the proportion (50%, 1.2 ng/mL) of urinary CLO to ΣNEOs in CF-Inner Mongolia was higher than those of other NEOs. The reasons for these patterns are unknown, but suggest differences in exposure profiles and NEOs usage, depending on the geographic region (Chen et al., 2019).

Interestingly, the annual global consumption of CLO was approximately 3 times lower than that of THM (M. Zhang et al., 2012); and the number of CLO-containing products ($n = 8$) registered for Chinese market was 10 times lower than that of THM-containing products ($n = 156$) (Shao et al., 2013). Furthermore, THM was ubiquitously present in vegetables and fruits collected in Hangzhou, China, with concentrations much higher than those of CLO (Lu et al., 2018). However, urinary CLO concentrations (range: 0.10 to 1.2 ng/mL) were higher than those of THM (0.080 to 0.45 ng/mL) in all sampling locations except for SJZ-Hebei, in our study. In vivo studies have showed that THM can be converted to CLO in insects (i.e., *Spodoptera frugiperda* larvae), mice and plants (i.e., cotton and rice) (Nauen et al., 2003; Casida, 2011). Several in vitro studies also indicated CYP3A4, the most abundant P450 in humans, can facilitate the conversion of THM to CLO (Casida, 2011; Han et al., 2018; Cimino et al., 2017). Thus, higher urinary CLO levels than those of THM might be explained by the metabolic conversion of THM in humans. Furthermore, median urinary CLO concentrations were higher than those of IMI in most sampling locations (Fig. 1). This pattern was unexpected, as global annual consumption of IMI was 4 times higher than that of CLO (Harada et al., 2016). CLO is more likely excreted in urine as unchanged compound than IMI did (Harada et al., 2016). Therefore, unexpectedly higher urinary CLO levels than those of IMI can be explained by compound-specific urinary excretion.

3.3. Age- and gender-related patterns of urinary NEOs

We examined age-related patterns of urinary NEOs levels (log-transformed values) across all participants (data not shown) by Pearson's rank correlation. No significant associations were found between age and urinary NEOs ($p > 0.05$). Furthermore, no significant differences in urinary NEO concentrations were observed among < 18, 18–50, and > 50 yrs. groups ($p > 0.05$, Mann Whitney U test) (Fig. 3).

However, we found significantly higher urinary levels of ΣNEOs in males than in females ($p < 0.05$; males vs. females = 1.4 vs. 0.98 ng/mL). Similarly, males had significantly higher urinary IMI ($p < 0.01$; 0.28 vs. 0.17 ng/mL) and ACE ($p < 0.01$; 0.019 vs. 0.010 ng/mL) concentrations than did females (Fig. 3). For all participants with urinary concentrations above the 90th percentile value for ACE (0.087 ng/mL), IMI (1.2 ng/mL), and ΣNEOs (3.8 ng/mL), sex was significantly associated univariately. Males were 7.0, 9.0, and 5.0 times more likely than females to have concentrations above the 90th percentile for ACE, IMI, and ΣNEOs, respectively. To remove the effects of gender distribution among sampling locations, we excluded data for GL-Guangxi, NC-Sichuan, and DT-Shanxi to examine gender-related patterns in NEO concentrations, due to the limited number of females (< 30% of participants) participated in these locations. Nevertheless, the urinary levels of ACE (males vs. females: 0.017 vs. 0.010 ng/mL), IMI (0.26 vs. 0.17), and ΣNEOs (1.2 vs. 1.0) remained significantly higher in males than in females ($p < 0.05$). Furthermore, this pattern was observed in participants of age groups < 18 and 18–50 yrs., but not for donors of > 50 yrs. No previous studies have reported significantly gender-related differences in urinary NEOs concentrations (Ueyama et al., 2014, 2015; Osaka et al., 2016; Kabata et al., 2016; López-García et al., 2017; Wang et al., 2015, 2017, Zhang et al., 2018). For example, no gender-related differences in urinary concentrations of 7 NEOs in children ($n = 223$) and adult group ($n = 52$) were found in Japan (Osaka et al., 2016; Ueyama et al., 2014), although urinary ΣNEOs (sum of 7 NEOs) concentrations (median: 5.2 vs. 4.0 nmol/g) in males were slightly higher than in females (Osaka et al., 2016). In general, males had higher dietary intake than that of females in China (Chinese Nutrition Society, 2013), which may result in higher levels of NEOs exposure by males. Another possible explanation of higher exposure in males is the gender-related metabolism of NEOs. However, the reasons for gender-related differences are still unclear, further study is needed.

3.4. Correlations and source analysis

Pearson's correlation analysis was used to test the relationships of urinary concentrations (log-transformed) of six NEOs. When all sampling regions were collectively considered, significantly positive correlations ($p = 0.025$ to 0.000) were found among all pairs of urinary NEOs with the correlation coefficients ranging from 0.135 (ACE vs. DIN) to 0.661 (THM vs. CLO) (Fig. 2). In general, urinary NEOs concentrations were significantly positively correlated with each other in most sampling locations (Table S3) when these relationships were examined for each location. NEOs are mostly used in rice and wheat seeds coating, and they are also used on other cereals, fruits and vegetables (Englert et al., 2017; Bass et al., 2015). Furthermore, NEOs are used in later phases of growth cycles of food-based plants, ornamental flowers, grasses and trees via drip, broadcast and foliar spray (Anderson et al., 2015; Choi et al., 2013). Limited studies indicated simultaneous existence of NEOs in vegetables, fruits and honey worldwide (Lu et al., 2018; Chen et al., 2014; Mitchell et al., 2017). Our results of relationships among NEOs suggest that the sources of human exposure to these

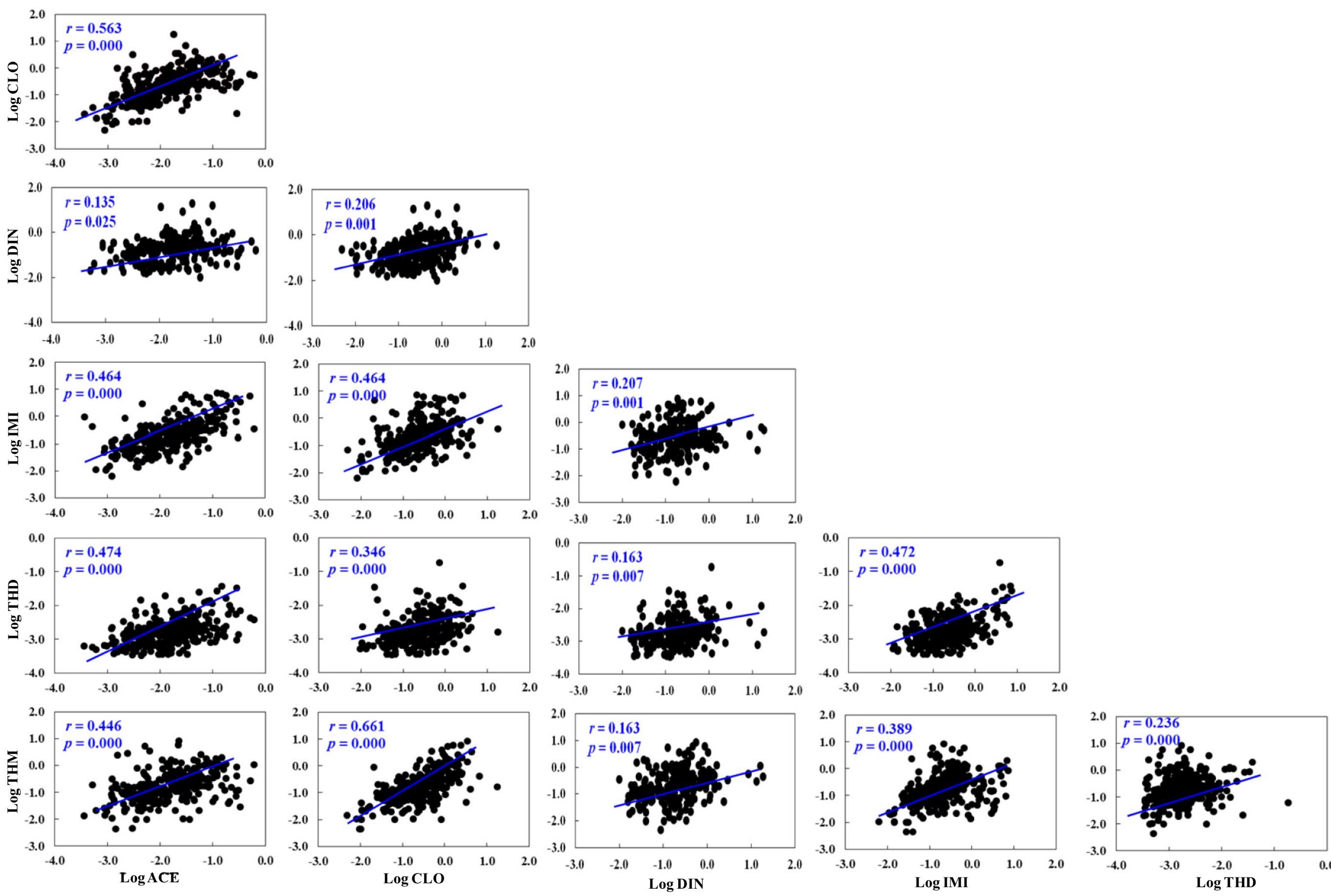

Fig. 2. Pearson correlations among individual neonicotinoid pesticide concentrations measured in urine samples collected in China. *We used* log*-transformed urinary concentrations for these analyses. Data on urinary NEOs in LS&SG-Tibet and YX-YN were excluded from the estimation of relationships between urinary DIN and other NEOs due to that DIN was not analyzed in samples from LS&SG-Tibet and YX-YN.*

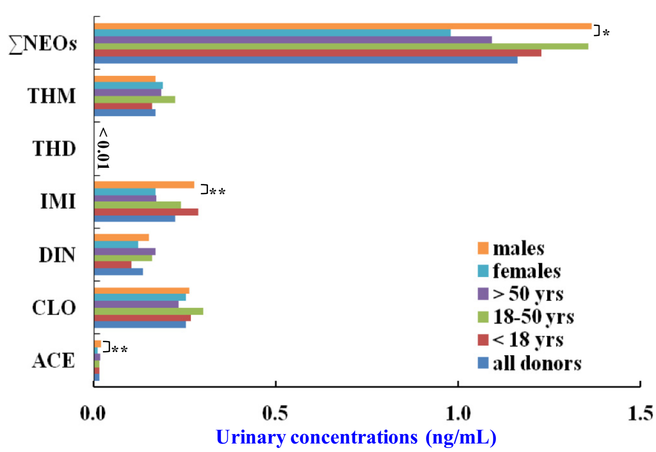

Fig. 3. Median concentrations of individual neonicotinoids (NEOs) and their sum concentration (ΣNEOs) in urine samples collected from China, as stratified by age and gender. *Due to DIN was not analyzed in Tibet and Yuxi-Yunnan, data on ACE, CLO IMI, THD and THM concentrations generated for both of sampling locations were excluded from age- and gender-related differences analysis; double asterisk (**) indicates significant gender-related difference with p value < 0.01; single asterisk (*) indicates significant gender-related difference with p value < 0.05; no significant differences were found among all three age groups; median concentrations of THD in all groups were < 0.01 ng/mL.*

insecticides are common in China. Importantly, correlations between concentrations of THM and CLO were strong (Fig. 2). As indicated above, CLO is a metabolic product of THM (Casida, 2011; Han et al., 2018; Cimino et al., 2017). Thus, in addition to daily intake via food (Chen et al., 2014) and drinking water (Klarich et al., 2017) consumption, CLO can originate from the metabolism of THM in human bodies.

3.5. Global comparison of urinary NEOs

The urinary concentrations of NEOs reported from various studies worldwide are summarized in Table 2 (Ueyama et al., 2014, 2015; Osaka et al., 2016; Kabata et al., 2016; López-García et al., 2017). Currently, most studies on urinary NEOs were from Japan (Ueyama et al., 2014, 2015; Osaka et al., 2016). Ueyama et al. (2015) measured concentrations of 7 NEOs in human urine from Japanese women between 1994 and 2011. The DRs of urinary NEOs increased significantly over this period with the DRs ranging from 6% (ACE) to 89% (THM) in year 2011. In another study from Japan, low DRs (0% to 58%) of NEOs in children' urine (n = 703) were observed. The DRs of NEOs in human urine from Japan were much lower than those found in China in this study (> 90% for all studied NEOs). This difference can be attributed to the higher LOQs (range: 0.03 to 3.6 ng/mL) of the Japanese study, which were two orders of magnitude higher than those of this study (ACE 0.007, CLO 0.015, IMI 0.02, THD 0.0005, and THM 0.02 ng/mL) (Osaka et al., 2016). However, widespread (DRs: 56% to 100%) occurrence of NEOs in human urine was found in adults (n = 52) living in Nagoya, Japan, when LOQs were lower (range: 0.05 to 0.36 ng/mL)

Table 2
Summary of urinary concentrations (ng/mL[a]) expressed as median and detection rates (in parentheses) of neonicotinoid insecticides in the general population from China with those reported for other countries.

Countries	Age (yrs)	N	ACE	CLO	DIN[c]	IMI	THD	THM	Sampling locations	Sampling date	Ref.
China	1–97	324	0.014 (96%)	0.24 (99%)	0.14 (96%)	0.21 97%)	0.0016 (92%)	0.15 (98%)	13 cities	2016–2017	This study
Japan	45–75	18	< 0.20 (6%)	< 3.6 (11%)	1.8 (75%)	< 0.90 (39%)	< 1.1 (33%)	0.91 (89%)	Kyoto city and surrounding areas	2011	(Ueyama et al., 2015)
Japan	45–75	17	< 0.20 (29%)	< 3.6 (6%)	0.5 (50%)	0.43 (52%)	< 1.1 (12%)	0.69 (88%)	Kyoto city and surrounding areas	2009	(Ueyama et al., 2015)
Japan	3	703	< 0.03 (12%)	< 1.1 (8%)	0.44 (58%)	< 0.31 (15%)	< 0.32 (0%)	< 0.22 (25%)	Nagoya city	2012	(Osaka et al., 2016)
Japan	> 18	52	0.02 (56%)	0.70 (96%)	2.3 (100%)	1.9 (96%)	0.14 (67%)	0.50 (100%)	Nagoya city	NA[e]	(Ueyama et al., 2014)
Sri Lanka[b]	NA[e]	20	< LOD (0%)	< LOD (0%)	< LOD (0%)	0.015 (85%)	Not analyzed[d]	< LOD(25%)	Medawachchiya and Girandurukotte	2011	(Kabata et al., 2016)
Spain	> 18	36	< 0.20(3%)	< 0.20(0%)	< 0.20(6%)	< 0.20(3%)	< 0.20(0%)	< 0.20(0%)	NA[e]	NA[e]	(López-García et al., 2017)

[a] 1.0 ng/mL = 1000 ng/L.
[b] No detailed information on LOD values was described in that study.
[c] Urinary level of DIN in China was generated based on 10 cities due to this compound was not analyzed in LS&SG-Tibet and YX-Yunnan.
[d] The corresponding NEOs were not analyzed in these studies.
[e] "NA" represents that information is not available.

(Ueyama et al., 2014). The median urinary concentration of ACE (China vs. Japan = 0.014 vs. 0.02 ng/mL) in China was similar to that found in Japan, whereas the median concentrations of CLO (0.24 vs. 0.70), IMI (0.21 vs. 1.9), DIN (0.14 vs. 2.3) THD (0.0016 vs. 0.14) and THM (0.15 vs. 0.50) in China were much lower than those reported in Japan (Table 2). It is worth to note that our median values were based on the data from 13 cities in China, whereas only one location (i.e., Nagoya city) was studied in Japan. As indicated above, NEO concentrations can vary among locations; the highest median values of ACE (0.10 ng/mL in GL-Guangxi), CLO (1.2 ng/mL in CF-Inner Mongolia), IMI (3.2 ng/mL in GL-Guangxi) and THM (0.45 ng/mL in MM-Guangdong) in some locations in China were higher than those found in Nagoya, Japan.

Low DRs of NEOs in human urine were also observed in Sri Lanka ($n = 20$; DRs: 0% to 25%) and Spain ($n = 36$; DRs: < 5%), attributed to high LOQ (0.20 ng/mL) and small sample size (Kabata et al., 2016; López-García et al., 2017). The median urinary concentrations of IMI (China vs. Sri Lanka = 0.21 vs. 0.015 ng/mL) in China were one order of magnitude higher than those found in Sri Lanka, and the median urinary levels of CLO (China vs. Spain = 0.24 vs. < 0.20) and IMI (0.21 vs. < 0.20) were higher than those observed in Spain. These findings indicate a high level of exposure to CLO and IMI in China.

3.6. Human exposure to imidacloprid

To evaluate exposure dose of target NEOs in humans, the daily intakes (DIs) of individual NEOs were estimated from the urinary concentrations (Table 3). There exists limited data on kinetics or metabolism of NEOs in the human body. Only for IMI, quantitative data on the excretion of metabolites in urine are available (Harada et al., 2016). Moreover, a human study showed short biological half-live ($t_{1/2}$: 1.5 day) of IMI (Ford and Casida, 2006). Based on the information, the DIs of IMI was estimated from its urinary concentrations by using the following equation (Eq. (1)) developed by Harada et al. (2016).

$$DI\,(\mu\text{g/day}) = \frac{U(N)}{r \times (e^{-\alpha \times 24(N-1)} - e^{-\alpha \times 24N})} \tag{1}$$

where $U(N)$ is the amount of IMI excreted in urine between $N - 1$ days and N days, r is the proportion excreted in urine, α is the elimination rates. In this study, the value of N is 1 day. The elimination rate (α) of IMI and r value were used 0.479/d and 0.133 (Harada et al., 2016).

The median (mean, range) DIs of IMI across all studied participants from China were 1.6 (4.1, < 0.02 to 55) μg/day, whereas the DIs of IMI varied by sampling locations with the median values ranging from 0.52 (YY-Hunan) to 24 (GL-Guangxi) μg/day (Table 3). Our results are much higher than those estimated for donors from Japan (0.19 μg/day). Another study reported by Lu et al. (2018) quantified total dietary intakes of 7 NEOs via fruits and vegetables with the values of 10.1 and 37.9 μg/day, respectively, in the United States and Zhejiang Province, China, indicating that fruits and vegetables are the major sources of human NEOs exposure.

We further calculated the DIs of IMI on the basis of body weight (μg/kg bw/day), and these values were compared against reference doses (RfD, the maximum daily oral dose of a pesticide that the U.S. EPA deems acceptable) (Table 3) (Lu et al., 2018). The median (mean, range) DI values on the basis of body weight were 0.034 (0.11, < 0.0003 to 2.1) μg/kg bw/day, and the maximum DI values were 30 times lower than the RfD value (57 μg/kg bw/day). These results suggest a low health risk from current level of exposure to IMI in China.

4. Conclusions

In summary, we measured six NEOs in Chinese urine samples collected across the nation for the first time in this study. Our results suggest a widespread exposure of Chinese to all six NEOs. Urinary levels of ΣNEOs were mainly accounted for by CLO (median: 0.24 ng/mL), IMI

Table 3
Estimated daily intakes of imidacloprid in China, calculated from measured urinary concentrations.

Locations	daily intake (μg/day)					daily intake (on body weight basis, μg/kg bw/day)[a]				
	Median	Min	Max	Mean	GM	Median	Min	Max	Mean	GM
All sites	1.6	< 0.02	55	4.1	1.4	0.034	< 0.0003	2.1	0.11	0.033
GL-Guangxi	24	0.81	55	24	15	0.41	0.032	2.1	0.69	0.38
CF-Inner Mongolia	2.0	0.17	29	5.0	2.6	0.034	0.0028	0.48	0.083	0.044
MM-Guangdong	2.7	0.68	14	3.5	2.6	0.05	0.013	0.23	0.063	0.049
NT-Jiangsu	2.0	0.69	5.0	2.2	1.9	0.033	0.011	0.084	0.036	0.031
DY-Shandong	2.5	0.32	17	3.6	2.5	0.066	0.0053	0.67	0.10	0.064
DT-Shanxi	2.2	0.23	15	2.9	2.0	0.041	0.0039	0.24	0.052	0.035
LS&SG-Tibet	1.0	< 0.02	4.9	1.6	0.98	0.021	< 0.0003	0.081	0.029	0.018
YX-Yunnan	0.79	0.17	22	1.7	0.78	0.021	0.0028	0.37	0.037	0.018
NC-Sichuan	0.94	0.047	3.5	1.0	0.58	0.016	0.0008	0.061	0.02	0.011
NC-Jiangxi	0.63	< 0.02	11	1.4	0.40	0.019	< 0.0003	0.78	0.068	0.012
YY-Hunan	0.52	0.11	5.9	1.2	0.67	0.018	0.0038	0.18	0.035	0.018
SJZ-Hebei	0.57	0.082	12	1.6	0.70	0.013	0.0014	0.20	0.041	0.017

[a] Data on body weight were from The International Commission on Radiological Protection (Valentin, 2002).

(0.21 ng/mL), THM (0.15 ng/mL) and DIN (0.14 ng/mL), which collectively accounted for 98% of ΣNEO concentrations. Significant sex-related differences ($p < 0.05$) in urinary ΣNEOs, IMI and THM were observed. Region-specific differences in urinary NEOs concentrations were observed within China.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105114.

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