Evaluation of Dermal Absorption Rate of Pesticide Chlorpyrifos Using *In Vitro* Rat Dermal Tissue Model and Its Health Risk Assessment

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All pesticides must be assessed strictly whether safe or not when agricultural operators are exposed to the pesticides in farmland. A pesticide is commonly regarded as safe when estimated dermal absorption amount is lower than the acceptable operator's exposure level (AOEL). In this study, dermal absorption rate of chlorpyrifos, a widely used organophosphate insecticide, was investigated using rat dermal tissue model. Chlorpyrifos wettable powder solved in water (250, 500 and 2,500 ppm) was applied to freshly excised rat dermal slices (341~413 μ m thickness) on static Franz diffusion cells at 32°C for 6 hours. After exposure period of 6 hours, and then washing-at residual amount of chlorpyrifos was analyzed in dermal tissues, tape strips, washing solution, washing swabs of receptor bottles and receptor fluids at 1, 2, 4, 8 and 24 hours. Chlorpyrifos was only detected in dermal tissue but not found in receptor fluid at each concentration and time point, and the absorption rate of 250, 500 and 2,500 ppm was 2.36%, 1.96% and 1.69%, respectively. The estimated exposure level of chlorpyrifos was calculated as 0.012 mg/kg bw/day. The health risk for farmers in this condition is a level of concern because the estimated exposure level is 12 times higher than AOEL 0.001 mg/kg bw/day. However, actual health risk will be alleviated than estimated because absorbed chlorpyrifos is not permeated into internal body system and only retained in skin layer.

Key Words: Dermal absorption; Chlorpyrifos; Health risk assessment; Exposure assessment; Pesticide; Agricultural operator

INTRODUCTION

In workplace, hazardous materials enter human body mainly via inhalation and skin contact. Pesticides have been also exposed to operators through contact to when they have been loaded, mixed or sprayed in farmland and human maybe sometimes showed toxic symptoms such as nausea, dizziness, anorexia and so on (Chae et al., 2001; Eddleston et al., 2008).

In the process of operator's health risk assessment, it is regarded as safe when the estimated exposure amount is lower than acceptable operator's exposure level (AOEL) that is driven from No Observed Adverse Effect Level (NOAEL) of chronic or sub-chronic toxicity and appropriate uncertainty factors. However, as most of toxicity tests were performed through oral route, the information of toxicity through dermal exposure and dermal absorption rate is very limited and hard to assess health risk through dermal exposure (Van Ravenzwaay and Leibold, 2004).

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Field operators get a majority of internal dose through dermal contact to pesticides and most pesticides have low to moderate vapor pressure. It was reported that more than 90% of total exposure is come from the dermal contact during application of agrochemicals (Finley et al., 1994). The extent and rate of dermal absorption is dependent upon a large number of biological and environmental factors including site and area of exposure, media, temperature, occlusion pattern, working practice, personal hygiene and so on. (Dosemeci et al., 2001).

OECD, USA EPA and EU EFSA have established standardized guidelines of dermal absorption tests including *in vitro* models of animal dermal tissues and human dermal tissues and *in vivo* rodent model (EFSA 2012; EPA 2007; OECD 2004). The *in vitro* rat dermal tissue model using Franz diffusion cell system has lots of advantages in risk assessment, which are providing insights into the relationships among dermal, compounds and formulation in relatively simple way and providing higher absorption rate compared with *in vitro* human dermal tissue model and *in vivo* rodents model, that means it is good for protection of operators' health with higher margin of safety (Ng et al., 2010).

Chlorpyrifos, O,O-diethyl-O-3,5,6-trichloro-2-pyridyl phosphorothioate, is a broad-spectrum organophosphorus pesticide that eliminates moths, fleas, termites and various pests in the house and in farm lands of crop, vegetables and fruits growing area (Griffin et al., 1999). It induces neurotoxicities by inhibition of brain and erythrocyte cholinesterase activity and maternal and fetal toxicities. The lowest NOAEL was determined as 0.1 mg/kg bw/day based on the neurotoxicity observed in 2-year dietary toxicity studies in rats and dogs, and developmental toxicity studies in rats. The acceptable daily intake (ADI) for humans and AOEL for agricultural operators was established as 0.001 mg/kg bw/day with safety factor 100 by NIAS Korea (2016) and EFSA (2016). However, NOAEL evaluated by WHO is 1 mg/kg bw based on neurotoxicity in rats, mice and dogs and 0.1 mg/kg bw for humans, and ADI was determined as 0.01 mg/kg bw/day (IPCS, 1999), which AOEL value is 10 times higher than AOEL and ADI of EU and Korea.

Various formulas of chlorpyrifos products have been introduced in the market and contain mainly 20~25% of

chlorpyrifos as an active ingredient in forms of wettable powder or emulsified concentrates. In the farm, 1,000 times diluted chlorpyrifos with water has been used. Therefore, farmers can be exposed to 20~25% concentrate solution as they load the product in water whereas contacted to 0.025% (250 ppm) solution during spraying it to fruits or crops.

In several human volunteer studies, it was reported that only a small fraction of the dermal applied chlorpyrifos was absorbed by about 1.35% absorption rate (Griffin et al., 1999; Nolan et al., 1984). However, those studies were performed with an undiluted solution of concentrated chlorpyrifos product.

This study was performed to investigate the dermal absorption rate of the actually diluted chlorpyrifos solution, to which farmers are exposed during spraying in farmland, using *in vitro* rat dermal tissue model and to evaluate the safety of chlorpyrifos product to farmers through comparison of the operators' estimated exposure level with AOEL of chlorpyrifos.

MATERIALS AND METHODS

The studies were conducted in accordance with the OECD Principles of Good Laboratory Practice (OECD, 2004) and EFSA Guidance (2012).

Test substances

25% chlorpyrifos in formulation of wettable powder was used as a test pesticide product. It is labeled that 500~1,000 times dilution solution with water is recommended when spraying to fruit trees of apple, pear, peach and tangerine for the elimination of pest moths and used under protection condition of wearing glasses, gloves, masks and protective clothing. Chlorpyrifos diluted solutions of 250 ppm, 500 ppm and 2,500 ppm were prepared by mixing the 25% product (250,000 ppm) with water and 17.7 µl of each solution was applied to rat dermal tissues (diffusion area: 1.77 cm²) mounted in Franz diffusion cell apparatus (Hanson 6-Cell Test System, USA).

Table 1. LC-MS/MS conditions for the analysis of chlorpyrifos

Column	Accucore XL - C ₁₈ column (150 ×4.6 mm, 4 µm)	
Column temp.	20°C	
Mobile phase	20 mM ammonium acetate: methanol (10:90, v/v)	
Flow rate	0.5 mL/min	
Injection volume	5 µL	
Autosampler temp.	10°C	
Detector (MS/MS)	Positive ion mode, MRM mode	
	Target Compound	Chlorpyrifos 351.9 → 199.9
	Fragment	70
	Collision energy	20 V
	Capillary voltage	3,500 V
	Gas flow	10 (L/min)
	Gas temp.	350°C
	Nebulizer	40 psi
	Nozzle voltage	450 V
	Sheath gas flow	10 L/min
	Sheath gas temp.	400°C
	Nebulizing gas	Nitrogen gas
	Collision gas	Nitrogen gas

Preparation of rat dermal tissues and application of test materials

Rat dermal slices (200~400 µm thickness) were excised from full thickness of dorsal skin using dermatome (Integra Lifesciences Padgett Model S, USA). The integrity of excised dermal was examined morphologically with hematoxylin-eosin staining, that is, excised dermal slice was mounted on glass slide and fixed with methanol followed by H-E staining. Excised skin epidermis was mounted on Franz diffusion cell apparatus with the external surface of the stratum corneum upper most and then examined under microscope (40 X) according to the method described by Desmedt et al. (2015). Diameter and area of the mounted skin was 1.5 cm and 1.77 cm², respectively. The receptor chamber was filled with 7 ml of 1:1 ethanol: phosphate-buffered saline, pH 7.4 placed on a magnetic stirrer at 32±2°C. Each test materials, 250, 500 and 2,500 ppm of chlorpyrifos, was applied at 10 µL/cm² as a single dermal application for 6 hrs. At least 3 replicates were used for each dose.

Sampling, analysis of chlorpyrifos and calculation of absorption level

After 6 hrs of chlorpyrifos application to dermal tissue, the tissue was washed with 100 µL of a mild soap solution (0.1% w/v) and dried with two cotton buds. Washing soap solution and two cotton buds were combined and retained for analysis of unpenetrated pesticide. Receptor fluid was taken at 1, 2, 4, 8 and 24 hrs after 6 hrs application of each concentration of chlorpyrifos and analyzed for chlor pyrifos by using LC-MS/MS. After sampling the last receptor fluid, any residual chlorpyrifos was wiped on the surface of receptor chamber by using cotton swabs. The washing cotton swabs were also retained for analysis. Residual chlorpyrifos retained on the corneal layer of dermal tissue after 6 hrs application was taken out using tape strips (3 M Highland) two times. The tape strips were also collected for analysis. The dermal tissue preparations exposed to each concentration were removed from the diffusion cells and solubilized. Aliquots of receptor fluid and dermal washing solution, receptor chamber swabs, dermal tape strips, and dermal tissue were

Fig. 1. ESI mass spectrum of chlorpyrifos (10 ppm).

pretreated and analyzed for chlorpyrifos by LC-MS/MS.

The dermal absorption amount of chlorpyrifos was represented as the sum of measurements of receptor fluid, receptor chamber washes, and dermal samples excluding measurements of tape strips 1 and 2, and dermal washing solution. The dermal absorption rate (%) was calculated by dividing the total absorption amount with application dose and then multiplying 100.

Chlorpyrifos analysis

Samples were placed into tubes containing 10 mL of acetonitrile, vortexed for 5 min to extract chlorpyrifos, and then 10 mL of n-hexane was added. The mixture was shaken vigorously for 5 min, and centrifuged for 15 min at 2,400 (xg), and then bottom layer of acetonitrile was taken. 1 mL of acetonitrile fraction was filtered with syringe filter (pore size 0.25 μ m), and 5 μ L of filtered extract was injected and analyzed for chlorpyrifos using LC-MS/MS (1290 Infinity-Triquadro 6460, Agilent Tech. USA) in positive ion and MRM mode.

Chromatographic separation was achieved on a C18

column (Accucore XL, 150 $\times$ 4.6 mm, 4 μ m). 5 μ L sample was injected using an auto-sampler. Mobile phase compositions of 20 mM ammonium acetate: methanol (10:90, v/v) at a flow rate of 0.5 mL/min were used. The column oven temperature was operated at 20 $^{\circ}$ C. The spectra of chlorpyrifos was recorded on collision energy 20 V on relative abundance of molecular ion 351.9 as well as fragment ion 199.9 in MS/MS (Table 1, Fig. 1).

The recoveries of the method were determined by spiking different known concentrations of reference chlorpyrifos standards 10, 25 and 50 ng/mL into 0.01 M PBS. The percentage recovery of each concentration was calculated by comparing the peak area of the spiked standards with that of the pure standards in five times replications. The recovery rate of chlorpyrifos was calculated as 89.8~107.5% at 5, 10, 25, 50, 100 and 250 ng/mL. In this method, the analytical level of detection (LOD) was calculated as 1 ng/mL and the level of quantitation (LOQ) as 5 ng/mL (Table 2, Fig. 2).

Fig. 2. Calibration curve of chlorpyrifos in samples.

Table 2. Accuracy of chlorpyrifos analysis method using LC-MS/MS

Theoretical concentration (ng/mL)	Peak area	Calculated concentration (ng/g)	Accuracy (%)	CV ^{a)} (%)
5	40,429	5.27	105.4	2.56
10	79,648	9.29	92.9	1.14
25	207,834	22.46	97.5	1.55
50	463,812	48.75	89.8	1.32
100	1,029,979	106.90	106.9	2.16
250	2,605,942	268.76	107.50	3.24

^{a)} CV(%) = SD/Mean × 100 (n=5)

Estimation of exposure level of chlorpyrifos to operators during spraying the pesticide

Exposure level of chlorpyrifos to operators spraying pesticide was obtained using software of Korea Predictive Operator Exposure Model (KO-POEM) developed by Rural Development Administration, Korea by modifying the UK-POEM program. For calculation of exposure level, speed sprayer application of wettable powder product (25% chlorpyrifos active ingredient in the product, 1,000 times dilution

when spraying) was carried out under personal protection by clothing gloves and mask RPE (FFP3) during mixing and loading the pesticide, and by gloves during spraying at work rate of 4,500 L/hectare. Operators are assumed to spray the pesticide for 6 hrs to 2 hectare of orchard per day. Dermal absorption rate was allocated as 25% when it was exposed to product (chlorpyrifos 25% wettable powder) during loading and mixing, the default value recommended by EU EFSA for products having active substance concentration higher than 5% other than no information available (EFSA,

Fig. 3. Rat dermal tissue excised by dermatome. H.E. (40 X). (A) before application; (B) after application.

2012). And the absorption rate obtained from this study was used as the value when exposed during spraying the diluted product (chlorpyrifos 250 ppm).

Risk assessment of chlorpyrifos product for health of operators

After get the estimated exposure levels to operators, the value was compared to the Acceptable Operator Exposure Level (AOEL) of chlorpyrifos, which was determined as 0.001 mg/kg bw/day based on the No-Observed-Adverse-Effect-Level (NOAEL, 0.1 mg/kg bw/day) derived from the inhibition of acetylcholinesterase of erythrocytes and brain in dogs and rats (EC, 2015). When estimated exposure level is higher than AOEL, the level of exposure is regarded as a concern of risk for pesticide operator's health.

RESULTS

The integrity of excised rat dermal tissue

Before the mounting of excised rat dermal tissue on Franz cell diffusion system, the integrity of tissue was examined morphologically by the H-E staining. The thickness of rat dermal tissues excised was 341~413 μm and composed stratum corneum, epidermis and part of dermis with no change in histology ($\times 40$) (Fig. 3).

Chlorpyrifos content in collected specimens

In the present study, the applied amount of each prepared solution of 250, 500 and 2,500 ppm was 4.4, 8.8 and 44 μg of chlorpyrifos, respectively. 88.0~89.1% of applied dose did not penetrate into the dermal tissue layer and washed out from dermal tissue after 6 hrs application. In addition, recovered chlorpyrifos not penetrated into corneal layer and taken out by strip tapes was 0.039 ± 0.004 , 0.071 ± 0.016 and 0.220 ± 0.025 $\mu\text{g/mL}$ at 250, 500 and 2,500 ppm, respectively. The absorbed chlorpyrifos was mainly distributed into dermal tissue and not permeated into receptor fluid, in which chlorpyrifos was not detected at each sampling time. Total absorbed level was 0.105 ± 0.027 , 0.170 ± 0.012 and 0.750 ± 0.033 at 250, 500 and 2,500 ppm, respectively. The absorption rate of chlorpyrifos in this *in vitro* test was $2.36 \pm 0.61\%$ at 250 ppm, $1.92 \pm 0.14\%$ at 500 ppm and $1.69 \pm 0.08\%$ at 2,500 ppm after 6 hrs application (Table 3).

Estimated exposure level of chlorpyrifos to pesticide operators and its risk

We estimated exposure level of chlorpyrifos of 1,000 times dilution solution (250 ppm) to operators during speed spraying for 6 hrs by using KO-POEM program and its level was calculated as 0.012 mg/kg bw/day (Table 4). The condition of exposure reflected actual situation of use; the pesticide operator uses speed sprayer wearing personal pro-

Table 3. Chlorpyrifos concentration in dermal tissues, tape strips, dermal surface washing solution, receptor fluids and receptor chamber washing cottons after application of each concentration of chlorpyrifos product for 6 hrs

Group	Chlorpyrifos content, µg/mL (% of application dose)								Receptor chamber washing swabs	Total Recovery (%)	Total absorbed ^a (µg)	Absorption rate (%)
	Dermal tissue	Strips 1 & 2	Dermal surface washing solution and swabs	Receptor fluid collected at each time after 6 hrs application								
				0 hr	2 hrs	4 hrs	8 hrs	24 hrs				
Chlorpyrifos 250 ppm (17.7 µl, 4.4 µg)	0.105±0.027 (2.36±0.61)	0.039±0.004 (0.88±0.10)	3.893±0.271 (88.0±6.1)	–	–	–	–	–	91.2 ±6.8	0.105 ±0.027	2.36 ±0.61	
Chlorpyrifos 500 ppm (17.7 µl, 8.8 µg)	0.170±0.012 (1.92±0.14)	0.071±0.016 (0.80±0.18)	7.794±0.371 (88.1±4.2)	–	–	–	–	–	90.8 ±4.5	0.170 ±0.012	1.92 ±0.14	
Chlorpyrifos 2,500 ppm (17.7 µl, 44 µg)	0.679±0.031 (1.53±0.07)	0.220±0.025 (1.53±0.07)	39.417±1.258 (89.1±2.8)	–	–	–	–	–	91.3 ±2.7	0.679 ±0.031	1.54 ±0.08	

Data are mean ± SD (n=3). ^a: amount in dermal sample + in receptor fluid + in receptor chamber washing swabs.
–: not detected for lower than LOD (1 ppb)

Table 4. Estimated exposure level of chlorpyrifos product to pesticide operators during speed spraying for 6 hrs and its risk to operator's health

Chlorpyrifos products	Dermal absorption rate	Estimated exposure amount (mg/kg bw/day)	AOEL of chlorpyrifos (mg/kg bw/day)	Estimated exposure amount/AOEL	Health risk
250 ppm (1,000× dilution)	2.36% when speed spraying Default 25% when pesticide product loaded and mixed	0.012	0.001	12	A concern level but the risk will be alleviated for chlorpyrifos is retained in dermal layer and does not penetrate into internal system

tection of clothing gloves and mask RPE (FFP3) during mixing, and loading the pesticide and wearing gloves during 6 hrs spraying for 2 hectare of orchard per day at work rate of 4,500 L/hectare. Dermal absorption rate when exposed to product (chlorpyrifos 25% wettable powder) during mixing and loading was allocated as 25%, the default dermal absorption rate recommended by EFSA when the concentration of active ingredient is lower than 5% other than no information available (EFSA, 2012). And 2.36% of dermal absorption rate obtained from this study was used when exposed during spraying the diluted products (chlorpyrifos 250 ppm).

The estimated exposure level was calculated to be 0.012 mg/kg bw/day, that is 12 times higher than the AOEL of chlorpyrifos (0.001 mg/kg bw) suggesting that the estimated level of exposure is a level of concern for operators' health.

However, this study also showed that chlorpyrifos applied to dermal tissue was retained in epidermis and not penetrated into receptor fluid, which leads an assumption that internal dose of chlorpyrifos via dermal route will be much lower than the estimated exposure level 0.012 mg/kg bw/day (Table 4).

DISCUSSION

Dermal exposure of pesticides during spraying can result in a variety of skin diseases, neuronal disorders or systemic toxicity in pesticide operators (Eddleston, 2008). Absorption of pesticides through the skin is major exposure route when mixing, loading and spraying the pesticide. The degree of pesticide hazard by dermal absorption is related

with the toxicity potency of the pesticide, duration of the exposure, pesticide formulation, spraying types and the absorption rate (Dosemeci et al., 2001).

The rate of dermal absorption of chemicals depends largely on the penetration potency through outer layer of epidermis called the stratum corneum which serves an important barrier function by blocking molecules from passing into the skin (Morgan et al., 2003). In this study, *in vitro* static Franz diffusion cell system mounted with rat dermal tissue was used to investigate the absorption rate of chlorpyrifos. Almost 90% of application dose was washed out or just retained on the outmost layer of stratum corneum which was removed by strip tapping out. Only 1.69 to 2.36% of applied chlorpyrifos was permeated into lower layer of epidermis but still not absorbed into receptor fluid, which means that absorbed chlorpyrifos can induce dermal problems but not impact on systemic toxicity.

Chlorpyrifos is rapidly metabolized to chlorpyrifos oxon by mixed-function oxidases via oxidative desulfuration and an electrophilic phosphooxathiiran intermediate in mammals and toxicologically significant compounds in animals are the parent compound and chlorpyrifos oxon. And 3,5,6-trichloro-pyridol (TCP), a metabolite formed at final step is nearly completely excreted mainly via urine within 48 hrs (IPCS, 1999).

The status of dermal used in *in vitro* Franz diffusion cell model was good in structural integrity but not presenting vivid metabolism. It is why only chlorpyrifos was measured and not any of metabolic intermediates in this study. OECD also pointed that the influence of metabolism is much less significant for *in vitro* dermal absorption experiment due to lack of dermal viability and reduced physiological functioning (OECD, 2004). Also, metabolism may not be an important consideration factor if the compound remains in the stratum corneum. It is reported that metabolism processes in the epidermis and upper dermis can make lipophilic compounds more hydrophilic and enhance the penetration of a chemical through the skin (Pelling et al., 1997). So, in case of lipophilic compounds that cross stratum corneum, metabolism to be a more hydrophilic intermediates becomes an important factor for internal absorption. It is a reason why chlorpyrifos was just remained in dermal layer and not

permeated into receptor fluid in this study.

Dermal absorption rate of chlorpyrifos was reported to be 1% in human study (EC, 2015) but 1.69~2.36% was found when 250~2,500 ppm chlorpyrifos diluted with 0.01 M PBS (pH 7.4) was applied to rat dermal tissue in this study. In main cases, absorption by rodent dermal is 3-fold higher than that by human dermal regardless chemical classes (Poet, 2000). The higher absorption in rodent skin may be due to differences in dermal appendages (e.g. hair follicles), different morphology of the individual dermal layers, or immunological or metabolic factors (EPA, 1992).

Van Ravenzwaay and Leibold (2004) also reported that rat dermal tissue was more permeable (mean difference 10.9 fold) to various substances, which octanol/water partition coefficient 0.7~4.5 and molecular weight 231~394.3. Thus, the safety margin of pesticide is fully enough when systemic exposure level of humans is estimated based on the results of an *in vitro* rat dermal tissue study. It means the *in vitro* rat dermal tissue model may serve as a first tier test.

Aggarwal et al. (2015) also presented that dermal absorption rates are highly influenced by pesticides formula, that is, dermal absorption rates were lower than 6% for liquid concentrates, 2% for solid concentrates, and 30% for all spray dilutions, irrespective of the active substance concentration. In this study, chlorpyrifos product was wettable solution and diluted 1,000 times with water for spraying. This study showed that dermal absorption rate of spray dilution of chlorpyrifos is much lower than the default absorption value 25% recommended by EFSA when no information was available.

OECD (2004) has recommended a mean mass balance recovery of the test substance between 90~110% in *in vitro* dermal absorption study and a range of 80~120% for volatile test substances or unlabeled test substances. According to the recommendation, the sum amount recovered in all analyzed samples should be almost similar to the amount of application. In this study, the recovery of analyzed chlorpyrifos by summing all amounts detected in dermal layer, strips, washing solution, receptor fluid and receptor washing swabs comparing with application amount was 90.8~91.3% which leads our results are verified.

The NOAEL of chlorpyrifos was evaluated as 0.1 mg/kg

bw/day based on rat long term oral toxicity of inhibition of acetylcholine esterase and rat developmental toxicity including an increased delayed ossification, reduced crown-rump length, reduced pup weight, increase in postimplantation loss and delayed sexual maturity. And AOEL was set as 0.001 mg/kg bw/day by application of safety factor (100) (EC, 2014).

The exposure level of chlorpyrifos to pesticide operators is required to be lower than the AOEL. This study showed the estimated exposure level was calculated to be 0.012 mg/kg bw/day, which means the exposure level is 19 times higher than AOEL. However, chlorpyrifos was just found in dermal layer and not in receptor fluid after 6 hrs exposure time which showed that chlorpyrifos has limited dermal penetration power and lower systemic toxicity when exposed via dermal contact.

Diffusion across the non-living outer layer of dermal tissue, the stratum corneum, functions as a rate-limiting step for percutaneous absorption. The absorption and diffusion of a chemical through the stratum corneum depends on chemical physico-chemical properties including molecular weight, water and lipid solubility, polarity, and state of ionization (Jung et al., 2007; Ha et al., 2007). It has been postulated that small molecules having both lipid- and water-soluble properties are the most readily absorbed through the lipophilic environment of the stratum corneum and hydrophilic environment of the epidermis. The diluted wettable solution of chlorpyrifos tested in this study is regarded to enter the stratum corneum corneal layer and then remained for it was not metabolized to be more hydrophilic that could permeate through aqueous epidermis layer.

As a conclusion, dermal absorption rate of chlorpyrifos was investigated using verified rat dermal tissue model and was 1.69~2.36%. The estimated exposure level to pesticide operators using the dermal absorption rate was 0.012 mg/kg bw/day. The value is 19 times higher than AOEL but the health risk is assumed to be much lower than that estimated risk for chlorpyrifos because chlorpyrifos was confined in dermal layer and not permeated into internal layer of receptor fluid.

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Conflict of interest

The authors declare that they have no competing interests.

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