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*Original Article*

# Multigenerational effects of neonicotinoids (acetamiprid, clothianidin) on growth, fertility and motility of nematode *C. elegans*

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**ABSTRACT** — Neonicotinoids are potent agonists of nicotinic acetylcholine receptors that exert insecticidal effects by causing abnormal excitation of the nervous system. Neonicotinoids and their metabolites effect in mammals, including humans, have become a concern. In the present study, we evaluated the effects of chronic exposure of two neonicotinoids, acetamiprid (ACE) and clothianidin (CTD), on *Caenorhabditis elegans*. We used 1, 10, 100, and 1000 μM solutions of nicotine, ACE, and CTD dissolved in 1% dimethyl sulfoxide (DMSO). Bioassays and motility tests, which are neurotoxicity assessments, were performed on the L1–L2 larvae of wild-type *C. elegans*. To evaluate the effect of exposure over multiple generations and the correlation between concentrations and generations, the same study was conducted on the second and third generations of the exposed group. The bioassay results showed concentration-dependent adverse effects: body length, maturity rate, and lifetime number of pups decreased for both ACE and CTD for the first generation. In a multi-generation study, the effect intensified with the progression of generations, and the toxicity of both ACE and CTD was cumulative. This effect was more pronounced in breeding studies. The motility test results showed concentration-dependent adverse effects, such as a decrease in the number of behaviors for both ACE and CTD in both tests for the first generation. In a multi-generation study, the effect intensified with the progression of generations, and this effect was more pronounced with ACE exposure. Thus, the chronic exposure to ACE and CTD may cause cross-generational adverse effects, especially on *C. elegans* reproduction and motion.

**Key words:** Neonicotinoid, Acetamiprid, Clothianidin, Multigenerational effects, *C. elegans*

## INTRODUCTION

Neonicotinoid is the generic name for chloronicotinyl pesticides, which are potent agonists of nicotinic acetylcholine receptors and exert insecticidal effects by causing abnormal excitation of the nervous system (Tomizawa and Casida, 2003). These responses are based on nicotine structure. Neonicotinoids have been registered as pesticides in approximately 120 countries and are the most widely used pesticides worldwide because of their strong

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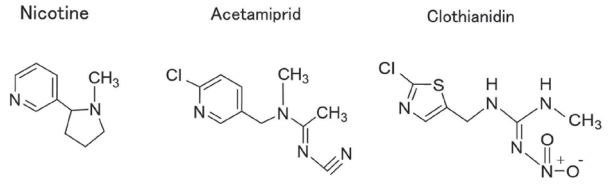


**Fig. 1.** Structures of Nicotine, Acetamiprid, and Clothianidin.

insecticidal activity against various pests and good levels of safety in vertebrates (Jeschke *et al*., 2011; Goulson and Kleijn, 2013). Seven types of neonicotinoids have been reported previously (Jeschke *et al*., 2011). Acetamiprid (ACE), which was released as a chloronicotinyl system in 1995, and clothianidin (CTD), which was released as a thianicotyl system in 2001, are widely used worldwide. The structures of ACE and CTD are shown in Fig. 1. Pesticide residue standards vary across countries as they are set according to the food crop intake of people in that country. However, even if the pesticide residue standards in the crop-exporting country are met, they may not meet the standards of the importing country. Therefore, international unification of the residue standards is being promoted. Neonicotinoid residue levels in fruits, vegetables, and tea leaves in Japan are ten times higher than those in other countries (Kimura-Kuroda *et al*., 2012a). Furthermore, it has been reported that ingestion of many domestic fruits and tea beverages for a long period of time causes symptoms of neonicotinoid poisoning, and high concentrations of neonicotinoid metabolites have been detected in the urine of some patients (Taira, 2012). Among them, Japanese tea leaves and polyethylene terephthalate- (PET) bottled beverage tea are reported to contain neonicotinoids at higher concentrations than those in other countries (Ikenaka *et al*., 2018; Takamoto *et al*., 2019). Thus, there are concerns regarding the health benefits of neonicotinoids in Japan.

Neonicotinoids, such as nicotine, bind to mammalian Neuronal nicotinic acetylcholine receptors (nAChRs); however, in binding inhibition experiments, binding to insect nAChRs was remarkably high compared to that in mammals, and the selectivity was estimated to be much higher. Owing to this difference in binding, the selectivity of neonicotinoids in mammals is weaker than that of nicotine, which is highly toxic to insects and mammals (Tomizawa and Casida, 2005). However, when considering toxicity to humans, it is important to note that neonicotinoids bind to mammalian nAChRs, even if they have low affinity, and the difference in binding experiments between insects and mammals is reported to be not directly related (Kimura-Kuroda *et al*., 2012b). Indeed, many cases of nicotine-like poisoning by neonicotinoids have been reported along with some deaths (Taira, 2012). Neonicotinoids have been shown to be causally related to the onset of attention deficit hyperactivity disorder in humans (Roberts and Karr, 2012) and reproductive disorders in mammals (Arican *et al*., 2020). In addition, in the ecosystem, there is a possibility that neonicotinoids are related to the loss of the homing instinct in honeybees (Cresswell, 2011; Whitehorn *et al*., 2012), Because of their high persistence in the soil, there is the possibility of biological effects across generations due to long-term exposure (Gibbons *et al*., 2015).

The nematode *Caenorhabditis elegans* is a multicellular organism approximately 1-mm long. Individuals of *C. elegans* are essentially self-fertilized clones with very few individual differences between them. They can be easily bred in the laboratory by feeding on *Escherichia coli* (Mitani, 2008). In addition, since *C. elegans* become adults after 3 days, it is possible to carry out multi-generational experiments in a short period of time. *C. elegans*, along with other soil organisms and protozoa, is involved in the circulation of materials in the soil and is suitable for studying their effects on ecosystems. Furthermore, the whole-genome sequence of *C. elegans* has been clarified, and it is considered as a suitable toxicity evaluation model for mammals because of its high homology with humans (Harlow *et al*., 2016). In recent years, neonicotinoid toxicity has been evaluated in *C. elegans* (Kudelska *et al*., 2017; Bradford *et al*., 2020); however, these reports were based on single-generation studies, and as pesticides are ingested over a long period by humans and ecosystems, it is important to consider their effects over multiple generations. In an earlier work, our group reported that the neonicotinoids imidacloprid (IMI) and dinotefuran (DINO) affect the growth, maturation, and reproduction of *C. elegans* for multiple generations (Sakaguchi *et al*., 2022).

This study evaluated the toxicity of ACE and CTD by using nicotine, a definitive nAChR agonist, for comparison. In addition, the multi-generational effects of neonicotinoids were assessed using bioassays (growth/maturation and reproductive effects tests) and motility test data from *C. elegans* exposed to ACE and CTD.

## MATERIALS AND METHODS

### Experimental animals

The wild-type nematode *C. elegans* (Bristol strain N2) was used. Live *E. coli* (DH5αFT) was used as food.

### Reagents

Acetamiprid (MW: 222.67, purity: 98.0%) and clothianidin (MW: 249.68, purity: 99.7%) standards were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Nicotine (MW: 162.23, purity: 99.0%) was purchased from Sigma-Aldrich (Tokyo, Japan). Each sample was dissolved in DMSO manufactured by Nacalai Tesque and used as the test solution.

NaCl, $Na_2PO_4$, Agar Powder, Tryptone, $CaCl_2$, Extract Yeast Dried, KCl, and $NaN_3$ were purchased from Nacalai Tesque. $KH_2PO_4$, KOH, cholesterol, $MgSO_4$, $C_6H_8O_7 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$, NaOH, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, and $Na_2EDTA$ were purchased from Wako Pure Chemical Industries. Sephadex G-25medium was purchased from GE Healthcare (Tokyo, Japan). All reagents were of special grade.

### Breeding of the nematode *C. elegans*

Mature nematodes were placed on nematode growth medium (NGM) plates and left for 3–4 days in a thermostatic chamber (20°C) to propagate by self-fertilization. On confirmation that the next generation of nematodes had reached maturity under a stereomicroscope, they were transferred to new NGM plates.

### Preparation and exposure of nematode suspensions

After culturing the propagated nematodes on NGM plates, only L1–L2 nematodes were isolated by passing them through a column packed with Sephadex G-25. Exposure solutions of each concentration were prepared using a liquid medium containing *E. coli* as the test medium and dispensed into 24-well plates at 0.5 mL/well. L1–L2 nematodes were added to the wells containing each exposure solution at a rate of 10 nematodes per well for the growth and maturation effect test and motility effect test, and one nematode/well for the reproductive effect test.

### Growth and maturation effect test

After L1–L2 nematodes were added to each exposure solution, they were incubated for approximately 50 hr at 20°C while shielded from light. After incubation, sodium azide was added and the body length of the nematodes and the percentage of nematodes with fertilized eggs were measured. Body length was used as an index to evaluate the effect on growth, and the percentage of nematodes with fertilized eggs was used as an index to evaluate the effect on maturation.

### Reproductive effects test

After L1–L2 nematodes were added to each exposure solution, incubation was initiated under shade at 20°C. After the first egg was laid in each exposure group, the parent nematodes were transferred to a new exposure solution daily, and this process was repeated until day 14 of exposure. The number of lifetime litters of each nematode was counted.

### Motility effects test

After L1–L2 nematodes were added to each exposure solution, they were incubated for approximately 24 hr at 20°C while shielded from light. After incubation, the number of body bends per 30 sec and thrashes per 60 sec was measured for each nematode.

### Multi-generational effects test

In this study, the method described by Goussen *et al*. (2013) was used as a reference. Following incubation, nematode suspensions were incubated at 20°C for 5 days and shielded from light. After incubation, only L1–L2 nematodes were isolated by passing them through a column packed with Sephadex G-25, and this was used as the second generation. Second-generation nematodes were provided in the quantities necessary for each test and each test was conducted. The remaining nematodes were cultured for 5 days in a new Petri dish with each concentration of culture medium, and third-generation nematodes were obtained in the same manner. Each test was repeated using the third-generation nematodes.

### Statistical analysis

Statistical analysis was performed using Microsoft "Excel Statistics Bell Curve for Excel" (Information Service Co. Ltd., Tokyo, Japan). A significant difference test was performed as an analysis of variance by Bartlett's test, and multiple comparative analyses were performed using the Williams test for growth and maturation effects, reproductive effects, and motility effects. The comparison target of the bioassay and the behavioral assay was the solvent control group, and the comparison target of the multi-generational effects test was the first generation of each concentration. All results obtained for the growth, maturation, and reproductive impact tests were presented as mean ± standard deviation. The level of statistical significance was set at $P < 0.05$.

## RESULTS

### Growth effects test

Nicotine significantly inhibited growth in a concentration-dependent manner at concentrations ≥ 1 μM in each generation. In the multi-generation effect test with 1000 μM nicotine, no results were obtained because the nema-

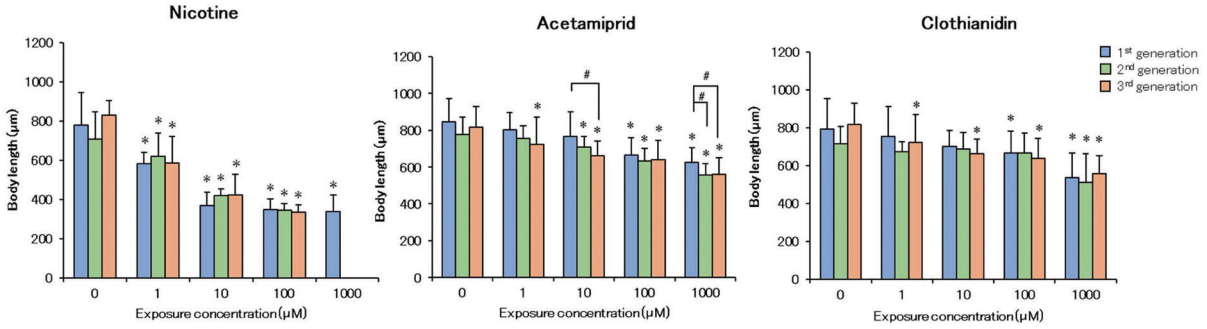


**Fig. 2.** Effects of nicotine and neonicotinoids on body length (μm) of multi-generational *C. elegans* after exposure in the growth effects test. n = 10. #1st generation vs. other generations: $P < 0.05$, *Control vs. other concentrations: $P < 0.05$.

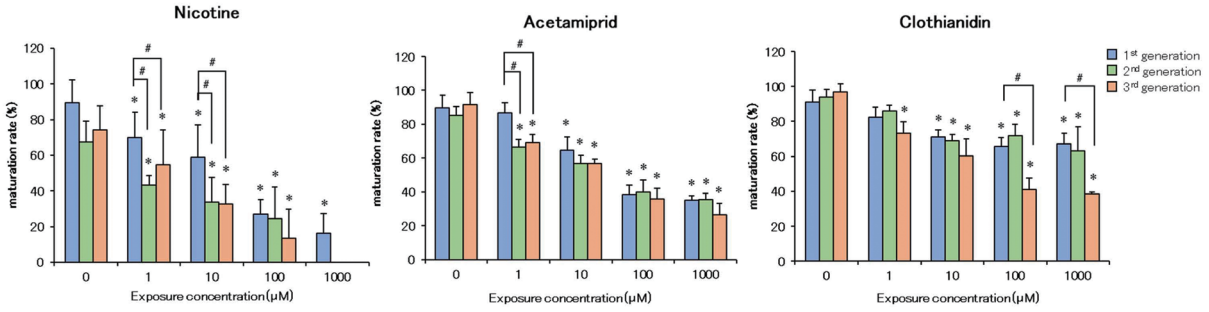


**Fig. 3.** Effects of nicotine and neonicotinoids on the maturation rate (%) of multi-generational *C. elegans* after exposure in the maturation effects test. 1 group: n = 10, 3 groups/each generation. #1st generation vs. other generations: $P < 0.05$, *Control vs. other concentrations: $P < 0.05$.

todes did not reproduce. In the first generation, neonicotinoids significantly inhibited growth in a concentration-dependent manner at ACE concentrations ≥ 100 μM and CTD concentrations ≥ 1000 μM. In the multi-generational effects test, the effect strengthened with the progression of generations, and ACE significantly inhibited growth in the third generation at 10 μM and in the second and third generations at 1000 μM. There was no significant difference between the generations in the CTD (Fig. 2).

## Maturation effects test

Nicotine significantly inhibited maturation in a concentration-dependent and intergenerational manner at concentrations ≥ 1 μM. In the multi-generational effect test, the effect tended to increase as the generation progressed, and the maturity rate decreased significantly in the second and third generations at 1 μM and in the second and third generations at 10 μM. In the multi-generation test with 1000 μM nicotine, no results were obtained because the nematodes did not reproduce. In the first generation, both ACE and CTD significantly inhibited maturation in a concentration-dependent manner at concentrations ≥ 10 μM. In the multi-generational effects test, the effect increased with the progression of generations, ACE significantly inhibited maturation in the second and third generations at 1 μM, and CTD significantly inhibited maturation in the third generation at 100 and 1000 μM (Fig. 3).

## Reproduction effects test

Nicotine caused a concentration-dependent and significant intergenerational decrease in the number of offspring produced over a lifetime at concentrations ≥ 1 μM. In the multi-generational effect test, the effect tended to increase as the generation progressed, and the number of offspring produced over a lifetime decreased significantly in the third generations at 1, 10, and 100 μM. In the multi-generation effect test with 1000 μM nicotine, no results were obtained because the nematodes did not reproduce. In the first generation, both ACE and CTD significantly inhibited the number of offspring produced over a life-

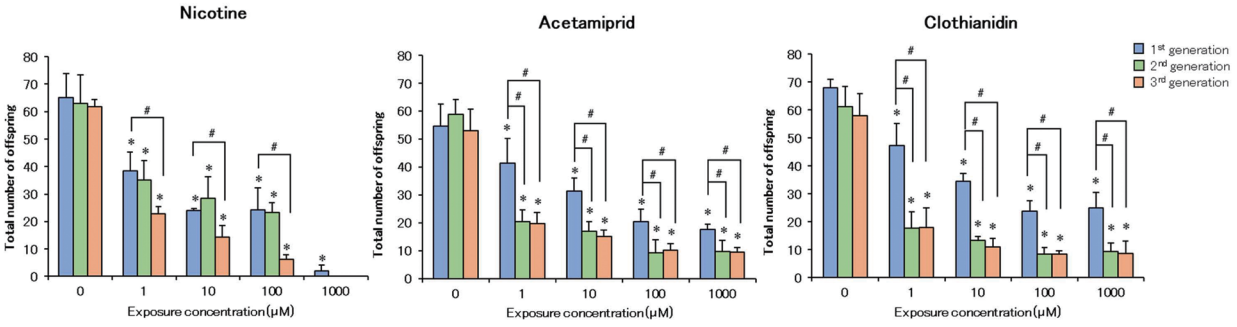


**Fig. 4.** Effects of nicotine and neonicotinoids on the total number of multi-generational *C. elegans* offspring after exposure in the reproduction effects test. n = 4. #1st generation vs. other generations: $P < 0.05$, *Control vs. other concentrations: $P < 0.05$.

time in a concentration-dependent manner at concentrations ≥ 1 μM. In the multi-generational effects test, the effect strengthened with the progression of generations, and both ACE and CTD significantly inhibited the number of offspring produced over a lifetime in the second and third generations at concentrations ≥ 1 μM (Fig. 4). In comparison with the results of the growth and maturation effect tests, the reproduction effect test showed that ACE and CTD caused a similar decrease in the number of lifetime offspring to nicotine after the second generation.

### Motility effects test

Nicotine significantly inhibited motility in a concentration-dependent manner at concentrations ≥ 1 μM in each generation. In the multi-generation effect test with 1000 μM nicotine, no results were obtained, because the nematodes did not reproduce. In the first generation, both ACE and CTD significantly inhibited body bend activity in a concentration-dependent manner at concentrations ≥ 1 μM. In the multi-generational effect test, both ACE and CTD became more effective as the generations progressed, and this effect was more pronounced at ACE (Fig. 5).

## DISCUSSION

In the growth and maturation effect tests, exposure to ACE and CTD inhibited growth and maturation in a concentration-dependent manner and progressed over generations. In a toxicity assessment of neonicotinoids using single-generation *C. elegans*, commercial IMI products in the United States were reported to adversely affect nematode growth and reproduction (Bradford *et al*., 2020). Furthermore, our previous studies revealed that IMI and DINO affect the growth, maturation, and reproduction of *C. elegans* for multiple generations (Sakaguchi *et al*., 2022). This study revealed that neonicotinoids ACE and CTD have the same adverse effects on *C. elegans* as IMI and DINO. Neonicotinoids affect the fertility of birds and spider mites (*Tetranychus urticae*) (Barati and Hejazi, 2015), and the results of the present study support these findings. In addition, the multi-generational experiment in this study confirmed a large multi-generational adverse effect similar to that of nicotine, particularly in reproduction. The adverse effects of ACE and CTD on reproduction became more pronounced across generations, indicating cumulative toxicity. This was similar to the significant reproductive effects of IMI and DINO observed in previous studies (Sakaguchi *et al*., 2022).

ACE has the characteristics of high osmotic transfer to plants, a high residual effect, and excellent egg-killing activity (Takahashi *et al*., 1998). In contrast, CTD has been put into practical use as a termite control agent in fields other than agriculture and is characterized by a wide insecticidal spectrum and strong insecticidal activity (Uneme *et al*., 2006). These two neonicotinoids are widely used worldwide, and there are concerns regarding the effects of chronic exposure to ecosystems and humans at low concentrations. The acceptable daily intake (ADI) is 0.071 mg/kg/day for ACE (Food Safety Commission of Japan Dec. 2014), and 0.097 mg/kg/day for CTD (Food Safety Commission of Japan Oct. 2014). Equal-dose toxicity is considered higher for ACE than for CTD. In this study, ACE tended to have a greater adverse effect than CTD. The standard values for residual pesticides are 30 ppm ACE and 50 ppm CTD for tea, which translates to ACE:134.7 μM and CTD:200.3 μM. In this study's reproduction effects test, a significant adverse effect was observed for both single and multiple generations at concentrations ≥ 1 μM, and ACE and CTD may affect ecosystem organisms even at low concentrations within the residual standard value.

In single-generation *C. elegans*, exposure to CTD

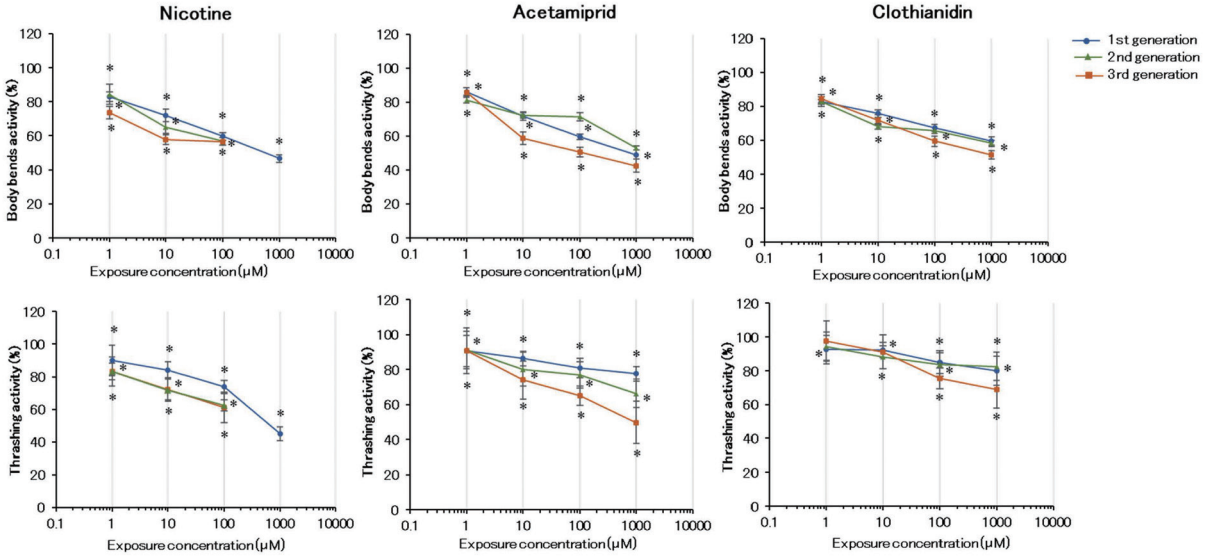


**Fig. 5.** Effects of nicotine and neonicotinoids on the thrashes and body bends of multi-generational *C. elegans* after exposure in the motility effects test. n = 10. DMSO exposure was 100%, and the motor activity at each exposure concentration was calculated. *Control vs. other concentrations: $P < 0.05$

has been reported to adversely affect motor function (Kudelska *et al*., 2017). In this study, similar results were obtained for CTD, and it was found that ACE also adversely affected the motor function of *C. elegans*. In addition, a multi-generational study showed that the adverse effects of ACE and CTD on motor function became more pronounced across generations and that toxicity was cumulative. Recently, behavior in *C. elegans* is being studied (Tsalik and Hobert, 2003). Decreased motor function in *C. elegans* has been associated with neurotoxicity (Anderson *et al*., 2004). Thus, it is clear from the results of this study that ACE and CTD show multi-generational neurotoxicity, similar to nicotine. In addition, compared with that of CTD, the motor function of ACE was significantly reduced, and there was concern regarding the strength of ACE neurotoxicity. Thrash, a nematode movement, is controlled by the balance between excitatory signals evoked when cholinergic motor neurons come into contact with muscle cells and inhibitory signals to the muscle tissue contralateral to GABAergic motor neurons (Petrash *et al*., 2013). These results indicate that exposure to the nAChR agonist neonicotinoids leads to an imbalance in excitatory and inhibitory signaling, leading to decreased motor function. Furthermore, cholinergic transmission of the body wall has been reported to not only regulate movement but also inhibit feeding behavior simultaneously (Izquierdo *et al*., 2021). In this study, the growth effect test showed a decrease in body length due to exposure to ACE and CTD, suggesting a relationship between motor function and growth in *C. elegans*.

It has been reported that Japanese adults consume an average of 0.53–3.66 μg/d of neonicotinoids in their diet (Harada *et al*., 2016). In addition, there are reports from other countries that Asians may be more exposed to neonicotinoids than non-Asians (Ospina *et al*., 2019). Both ACE and CTD have been frequently detected in Japanese tea leaves and PET bottles (Ikenaka *et al*., 2018; Takamoto *et al*., 2019). Furthermore, in recent years, it has been reported that ACE metabolites in the urine of low-birth-weight infants have been detected at a high rate due to the transfer of ACE from the human mother to the fetus (Ichikawa *et al*., 2019). There is growing interest in human toxicity due to chronic exposure to neonicotinoids. Neonicotinoids have been reported to act as agonists of α2β4nAChR in humans (Li *et al*., 2011). Human nAChRs are composed of five transmembrane subunits, and neuronal nAChRs contain various combinations of the α2-10 and β2-4 subunits (Kimura-Kuroda *et al*., 2012a). These proteins are also expressed in various tissues other than those of the nervous system. Insect nAChRs are composed of the same five αβ subunits as mammalian nAChRs, and their ACh binding sites are very similar to those of mammalian nAChRs (Crossthwaite *et al*., 2017). Insects have 10–12

nAChR genes and *C. elegans* has at least 27 genes (Sattell, 2009). The results of this study using *C. elegans* cannot be applied directly to humans but may indicate potential toxicity in humans. This study revealed the cross-generational adverse effects of ACE and CTD, and there was concern about their toxicity in humans due to chronic exposure to neonicotinoids.

However, the effects of neonicotinoids on ecosystems and humans are not yet fully understood. This study showed that a concentration within the residual standard value may adversely affect ecosystem organisms. In particular, chronic exposure to neonicotinoids has been suggested to have cross-generational adverse effects on reproduction. Furthermore, ACE may be as neurotoxic as nicotine due to chronic exposure. Therefore, it is necessary to conduct a comprehensive toxicity assessment, including genetic analyses, such as RNA sequencing and real-time PCR, in the future. In addition, our *C. elegans* toxicity assessment methods (including growth, maturation, reproduction, motility and multi-generational test) have the potential to provide a new and comprehensive toxicity assessment of chemicals.

**Conflict of interest----** The authors declare that there is no conflict of interest.

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