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Cyp35a2 gene expression is involved in toxicity of fenitrothion in the soil nematode *Caenorhabditis elegans*

Ji-Yeon Roh, Jinhee Choi *

School of Environmental Engineering, Graduate School of Energy and Environmental System Engineering, University of Seoul, 90 Jeonnong-dong, Dongdaemun-gu, Seoul 130-743, Republic of Korea

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ABSTRACT

In this study, the effect of organophosphorous (OP) pesticide, fenitrothion (FT), on the non-target organism was investigated using the soil nematode, *Caenorhabditis elegans*. Toxicity was investigated on multiple biological levels, from organism to molecular levels, such as, immoblity, growth, fertility, development, acetyl cholinesterase (AChE) activity and stress-response gene expressions. FT may provoke serious consequences on the *C. elegans* population, as it induced significant developmental disturbance. As expected, FT exposure inhibits AChE activity of *C. elegans*. The increased expression of the cytochrome p450 family protein 35A2 (*cyp35a2*) gene was also observed in FT exposed worms. To experimentally demonstrate the relationships between organism-level effects and the *cyp35a2* gene expression in FT-exposed *C. elegans*, the integration of the gene expression with biochemical-, and organism level endpoints were attempted using a *C. elegans cyp35a2* RNA interference (RNAi) and *cyp35a2* mutant (*gk317*). The 24 h-EC50s of *C. elegans* on FT exposure were in the order of *cyp35a2* RNAi in *cyp35a2* mutant (*gk317*) > *cyp35a2* mutant (*gk317*) > *cyp35a2* RNAi in *wildtype* (*N2*) > *wildtype* (*N2*). The higher EC50 values of *cyp35a2* RNAi and *cyp35a2* mutant (*gk317*) compared to that of *wildtype C. elegans* strongly supported that *cyp35a2* gene plays an important role in the toxicity of FT towards *C. elegans*. The experiments with *cyp35a2* RNAi also indicated that the development disturbance and decreased AChE activity, which were observed in FT exposed *wildtype C. elegans* were significantly rescued in the *cyp35a2* RNAi *C. elegans*. Overall results suggest that the *cyp35a2* may be an important gene for exerting FT toxicity in *C. elegans*.

1. Introduction

Pesticides are globally used in agriculture and may enter environments through spray drift or runoff events, drainage or leaching, and thus have been persisted in the surface or ground waters and in the soil (Cerejeira et al., 2003; Rice et al., 2010). The accumulation and persistence of pesticides in the environment constitute a threat to biological life in many wildlife animals, including human. For the management of pesticide use, the potential effects of pesticides need to be evaluated using non-target organisms at various levels of biological organization. As a combination of tests at different organizational levels, such as the molecular, biochemical, organism and population levels, allows the assessment of pesticides based on specific modes of action and ecologically relevant parameters. Fenitrothion (O,O-dimethyl O-4-nitro-m-tolyl phosphorothioate, FT), is an widely used organophosphorous (OP) insecticide. It is metabolically activated by cytochrome P450 isoenzyme superfamily to their oxon metabolites to be effective inhibitors of acetylcholinesterase (AChE), which causes neurotoxic effects due to the accumulation of the neurotransmitter acetylcholine in cholinergic synapses of both vertebrates and invertebrates organisms (Ricciardi et al., 2006; Damásio et al., 2007; Forcella et al., 2007; Sebire et al., 2009).

The nematode *Caenorhabditis elegans* is an excellent model organism for research on and assessment of environmental contaminants, particularly, for the study of the ecotoxicological relevance of chemical-induced molecular level responses, as the comprehensive knowledge of the genome of *C. elegans* and functional genomics tools allow for a clearer insight into the operation of toxic mechanisms initiated by chemicals acting upon organisms, mechanisms that can have adverse effects at the population level.

In this study, toxicity of FT was investigated on *C. elegans* using different biological responses that ranged from the molecular level to the population levels. Acute toxicity tests were performed by estimating the 24 h median effect concentrations (EC50s); and subsequently, the responses of the FT on the growth, fertility and development of *C. elegans* were investigated. Acetyl cholinesterase

* Corresponding author. Address: Faculty of Environmental Engineering, College of Urban Science, University of Seoul, 90 Jeonnong-dong, Dongdaemun-gu, Seoul 130-743, Republic of Korea. Tel.: +82 2 2210 5622; fax: +82 2 2244 2245.
E-mail address: jinhchoi@uos.ac.kr (J. Choi).

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(AChE) activity and stress response gene expression were measured as biochemical-, and molecular level indicators, respectively. To understand the relationships between organism-level effects and the CYP450 activation in FT-exposed *C. elegans*, the integration of the *cyp35a2* gene expression with biochemical-, organism-level endpoints were attempted using *C. elegans cyp35a2* RNA interference (RNAi). RNAi is widely used technology for gene function study, however its application is still very limited in ecotoxicology so far. In this study applicability of *C. elegans* RNAi for biomarker discovery was tested in ecotoxicological context.

2. Materials and methods

2.1. Organisms

The wild-type *C. elegans* Bristol strain N2 and *cyp35a2* mutant (*gk317*) was maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* strain OP50, at 20 °C, using the standard method previously described by Brenner (1974). Young adults (3 days old) from an age-synchronized culture were used in all the experiments.

2.2. Sample preparation

Six types of endpoints (mobility, growth, fertility, development, acetycholinesterase activity and gene expression) were assessed in the nematode exposed to FT. FT (Riedel–deHaen, Seelze, Germany) was dissolved in acetone. For each concentration and a control, three independent analyses were conducted for all the test types. FT concentrations in the K-medium (0.032 M KCl and 0.051 M NaCl; Williams and Dusenbery, 1990) were nominal values.

2.3. Gas chromatography – mass spectrometry analysis for the quantitative determination of fenitrothion

Concentrations of FT in the testing media were determined using the gas chromatography method. The extraction of FT from the water sample was performed using Solid Phase Extraction (SPE) method (Horizon technology, Salem, NH, USA), with acetone and MTBE as the extraction solvent. The sample analysis was carried out with an Agilent gas chromatography 6890 (Agilent technology, USA) using a NPD (Nitrogen/Phosphorous Detector) equipped with a DB-5MS fused-silica capillary column (30 m × 0.25 mm internal diameter, film thickness 0.25 μm, Agilent, USA).

2.4. Immobility, growth, fertility and development

Immobility, growth and fertility toxicity tests were performed as described previously (Roh et al., 2006). Briefly, following the 24 h exposure to FT, worms were analyzed for immobility, growth and fertility. Immobility was investigated by counting the numbers of worms that did not move but showing the response when were touched by platinum wire. The median effective concentration (EC50) was estimated using immobility as an endpoint. Growth was investigated by measuring the body length of worms and fertility was investigated by counting the eggs of each worm through the microscopic inspection. Fifty worms were examined per treatment for each three experiment.

The effects of pesticides on the development of nematodes were investigated by identifying the life stage of *C. elegans*, such as an egg, from first to third stage larva (L1–L3), forth stage larva (L4) and an adult, at regular time intervals, until 96 h after treatment. To obtain the F1 generation, one worm in the L4 stage from an age-synchronized culture (P generation) was introduced in the test medium. The total number of worms at each stage was determined for the F1 and F2 generations by observing the *C. elegans* under an optical microscope. The results were expressed as the percentage of each stage.

2.5. Acetylcholinesterase activity

Acetylcholinesterase activity was measured with the use of the method described by Roh and Choi (2008). Pooled young adults (about ~1000) were collected 24 h after treatment from control and experimental tanks and pooled for AChE measurements. Young adults were homogenized in Tris–EDTA buffer and the crude homogenate was centrifuged at 4 °C. The resulting supernatant (post-mitochondrial fraction) was used to measure the activity of AChE using Evolution 60 UV–Visible spectrophotometers (Thermo Scientific, Waltham, USA).

2.6. Quantitative real-time PCR

Following the 24 h incubation with exposure to sublethal concentrations of FT, worms were harvested for the analysis of gene expression using quantitative real-time PCR (polymerase chain reaction), as described previously (Roh et al., 2009b). Briefly, PCR method was used with IQTM SYBR Green SuperMix (Bio-Rad, Hercules, CA, USA), using a Chromo4 Real-Time PCR detection system (Bio-Rad). The primers were designed based on the sequences retrieved from the *C. elegans* database (www.wormbase.org; Supplementary table 1).

2.7. RNA interference

The RNAi feeding was performed, as previously described by Roh et al. (2010). The RNAi bacteria were induced for 48 h at room temperature for dsRNA expression. We added about 10, L1–L3-stage animals, onto the plate seeded with RNAi bacteria and incubated at 20 °C. After 36–40 h, worms were transferred to another large-scale plate seeded with the same RNAi bacteria and then the worms were allowed to grow to adults and to lay eggs. When worms grown in full, adults were removed by the age-synchronized culture method. Each egg was seeded to new, freshly prepared RNAi feeding plates. To evaluate the efficiency of the dsRNA feeding, more than 1000 worms were assessed by semi-quantitative PCR.

2.8. Data analysis

Median effect concentrations (EC50s) were derived through Probits analysis. The statistical differences between the control and treated worms were determined with the aid of the parametric *t* test using SPSS 12.0 KO (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

The multilevel biomarker approach has been widely used to determine the general health status of an organism in pollutant biomonitoring programs. However, plausible mechanistic linkage between the alteration observed at the molecular level and an adverse effect at the organism level needs to be demonstrated to validate a biomarker, as linking molecular changes with relevant organism level responses will improve the predictive powers of toxicity tests based on molecular responses. Well established functional genomics tools make *C. elegans* an excellent model for development of mechanism-based biomarker in toxicity testing and risk assessment.

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To select exposure concentrations for *C. elegans* toxicity assay, worms' sensitivity to FT exposure was tested using immobility as an endpoint. The 24 h-median effect concentration (EC50) of FT in *C. elegans*, was 0.501 mg L^{-1} (with 90% confident levels of) and based on this, three concentrations – corresponding to 1000^{-1}, 100^{-1} and 10^{-1} of the 24 h-EC50 – were selected as the exposure concentrations of FT toxicity analysis (i.e. 0.5, 5 and 50 μg L^{-1}).

Prior to the toxicity analysis, to confirm the FT exposure level in the test medium, a quantitative analysis on the FT residue was conducted (Fig. 1). The nominal concentration of FT in the test media at the beginning of the kinetics experiment was 50 μg L^{-1}. The results of the residue analysis for the 96 h-long study showed that the FT concentration in the medium rapidly decreased during the first 3 h, with less than 50% of the initial amount remaining after 48 h, but then reached about 20% of the initial concentration by the end of the experiment (96 h).

Physiological-level alterations, such as growth, reproduction, feeding, movement, lifespan and behavior, have been widely used as endpoints for chemical-induced toxicity testing in *C. elegans*, and they are broadly accepted reliable test parameters (Kumar et al., 2010; Pasco et al., 2010; Wang et al., 2010). In this study, growth, fertility and development (Supplementary Figs. 1 and 2A) were investigated to obtain comprehensive information on the toxicity of FT toward *C. elegans*. No statistically significant change was observed on the growth or fertility of nematodes estimated in *C. elegans* 24 h after exposure to FT. 24 h fertility test only provides a snapshot of the reproductive output and may not fully reflect embryonic lethality and delays in reproductive peaks. Such information, however, can be obtained from the 96 h-long development experiment. The effects of FT on the development kinetics of nematodes were therefore investigated by identifying the life stage of *C. elegans* for 96 h (Fig. 2A). In the control, 48 h after the beginning of the experiment, about half of the population was in the L1–L3 stage; and at 96 h, the eggs of the F2 generation accounted for about 70% of the total population. FT treatment provoked serious effect on the worm's development. At 0.5 and 5 μg L^{-1} FT treatment, the eggs of *C. elegans* from the initial worms (P generation) could not complete their development and failed to develop to the adult stage (F1). Thus, no F2-generation eggs appeared. At the highest FT exposure (50 μg L^{-1}), the worms in the L4 stage introduced at the beginning of the experiment (P) survived for 36 h, but failed to develop further; therefore, resulting in complete failure in the nematode's development. Of the three ecotoxicity batteries tested (growth, fertility, development), the most notable result was observed in the development kinetics by the full life cycle assay. The full life cycle assay seems to generate robust and population-relevant endpoints, which can provide an integrated approach for the prediction of the population responses of nematodes to the pesticide (Lopes et al., 2008; Roh and Choi, 2008). FT exposure affected the development during the F2 generation, suggesting serious consequences on the population of *C. elegans*.

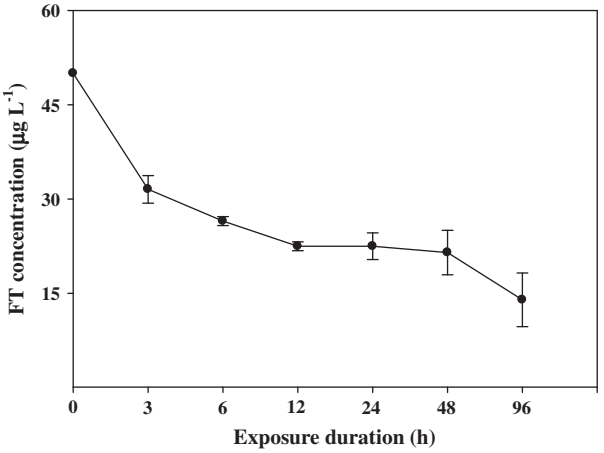

Fig. 1. Kinteitcs of FT concentration in test medium. Concentrations of FT in the testing media were determined using the gas chromatography method. (mean ± standard error of mean, number = 3).

Subsequently, AChE activity (Supplementary Fig. 2) and the stress response gene expression (Supplementary Fig. 3) were evaluated as molecular/biochemical level indicators, on young *C. elegans* adults exposed to FT for 24 h. As AChE is involved in the toxic mechanism of OP compounds, seriously decreased AChE activity was naturally observed in FT exposed worms. Genes covering a wide range of stress responses and defense systems, such as stress proteins, antioxidant enzymes and monooxygenase enzymes, were selected as potential responsive genes to FT exposure in *C. elegans*; these being heat-shock protein (*hsp-16.1*, *hsp-16.2* and *hsp-16.48*), metallothionein (*mtl-1* and *mtl-2*), vitellogenin (*vit-2* and *vit-6*), the cytochrome p450 family protein 35A2 (*cyp35a2*), superoxide dismutase-1 (*sod-1*), catalase-2 (*ctl-2*), the *C. elegans* p53-like protein (*cep-1*) and the apoptosis enhancer protein (*ape-1*). Fenitrothion induced *hsp-16.2*, *cyp35a2* and *ape-1* gene expressions; whereas, it led a decrease in *vit-2* gene expression. In particular, the induction of the *cyp35a2* gene expression was significant (Fig. 3).

The increased expression of the *cyp35a2* gene on FT exposure occurred concomitantly with inhibition of AChE and development failure, which suggests that the increase in the expression of this gene might lead to physiological consequences. The reduced development capacity may be explained as a part of the defense and/or compensatory mechanism to metabolize the toxicity induced by FT exposure. Although gene expression as an environmental stress response provides high sensitivity and; thus, can act as an early warning signal, relating such a molecular level response to physiological/ecological relevant effects represents a substantial challenge. To investigate the correlation between a validated toxicity endpoint (e.g., growth, fertility and mortality) and upstream-induced gene expression, in our previous study with metal exposure, statistical tests (i.e. correlation tests) were used, which revealed significant correlations between the stress-response gene expression and indicators from higher biological organization on metal exposure (Roh et al., 2006), however, a correlation analysis cannot provide any causal relationship between these parameters. *C. elegans* functional genomics can reveal the physiological meaning or relevance of altered gene expressions and; thus, help identify the mechanism based biomarkers. Recently, *C. elegans* mutant strains were used to evaluate the toxicities of cadmium and silver nanoparticles (Roh et al., 2009a,b) as well as RNAi for toxicities of CeO_2 and TiO_2 nanoparticles (Roh et al., 2010). Therefore, functional analysis was conducted on the toxicity of FT in *C. elegans*, in an attempt to reveal the role of *cyp35a2* gene expression in FT toxicity using *cyp35a2* RNAi. *cyp35a2* gene knock down by RNAi was confirmed by a decrease in the detectable band during PCR (Supplementary Fig. 4).

The 24 h-EC50s of *C. elegans* on FT exposure were then re-estimated on *cyp35a2* RNAi, *cyp35a2* mutant (*gk317*) and *cyp35a2* RNAi in the *cyp35a2* mutant (*gk317*) (Table 1), and found to be 0.991, 1.403 and 3.275 mg L^{-1}, respectively, which were 2, 3 and 6 times higher than those estimated in FT exposed *wildtype* (*N2*) *C. elegans*. The higher EC50 values of *cyp35a2* RNAi and *cyp35a2* mutant (*gk317*) compared to that of *wildtype C. elegans* strongly supported that *cyp35a2* gene plays an important role in the toxicity of FT towards *C. elegans*. Interestingly, the EC50 values was in the order of *cyp35a2* RNAi in *cyp35a2* mutant (*gk317*) > *cyp35a2* mutant (*gk317*) > *cyp35a2* RNAi in *wildtype* (*N2*) > *wildtype* (*N2*), which suggests that RNAi in the mutant was more efficient in reduction of

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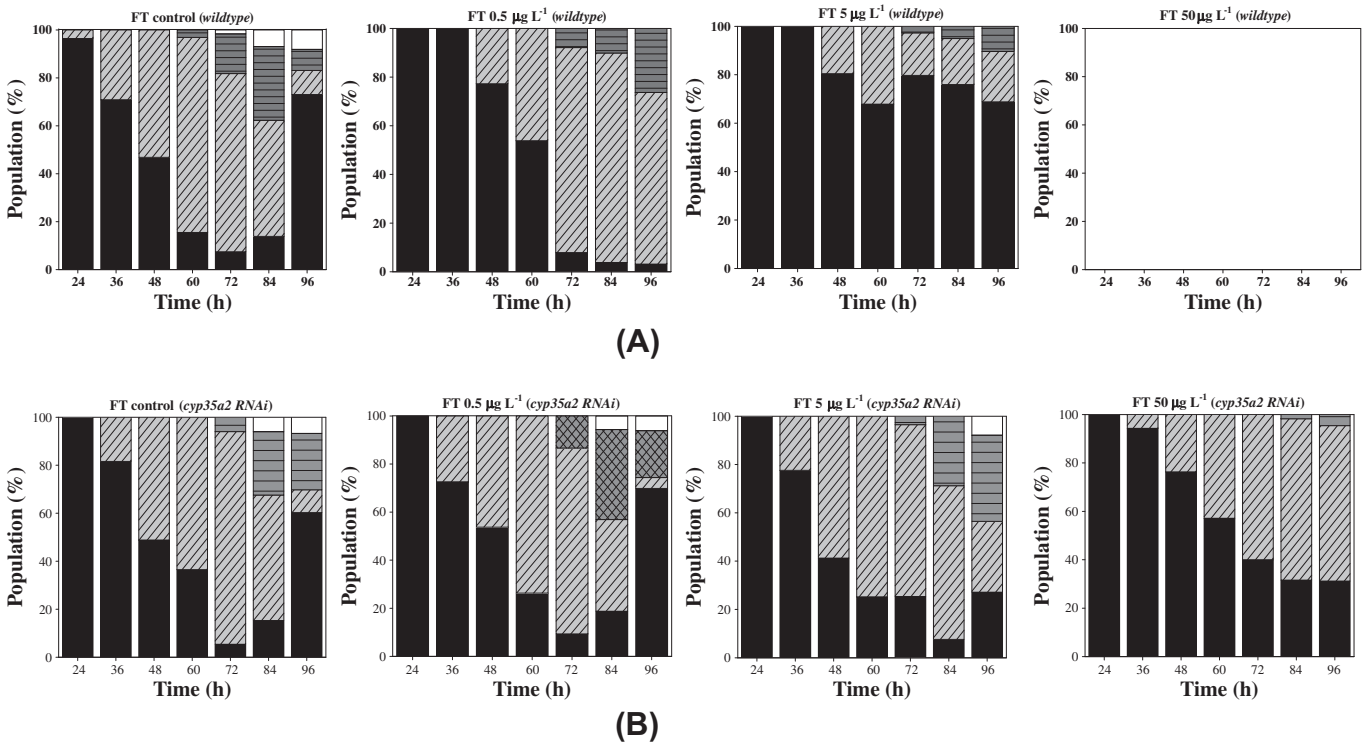

Fig. 2. Kinetics of population (development parameters) examined in *wildtype*(A) and *cyp35a2* RNAi(B) *C. elegans* exposed to FT for 96 h. The effects were investigated by counting the by identifying the life stage of *C. elegans*, such as an egg, from 1st to 3rd stage larva (L1–L3), 4th stage larva (L4) and an adult, at the regular time internals, until 96 h after treatment. The results were expressed as the percentage of each stage (■ egg, ▨ L1–L3, ▤ L4, □ adult).

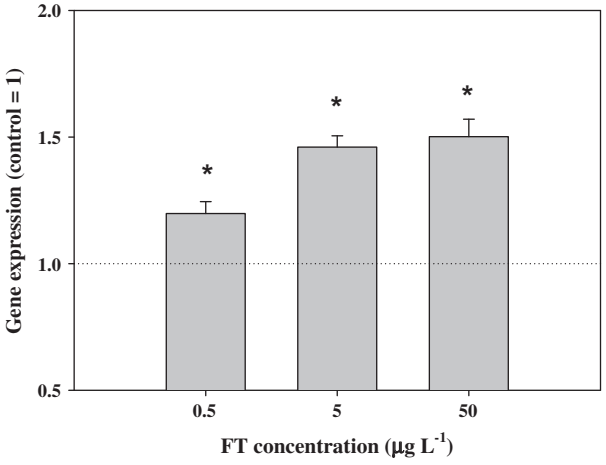

Fig. 3. Quantitative analysis on the expressions of *cyp35a2* gene in *wildtype C. elegans* exposed to FT for 24 h using real time-PCR (qRT-PCR). Gene expression was normalized using actin mRNA and data are presented in arbitrary unit compared to control (control = 1, replicates number = 3; mean ± standard error of the mean; $^{*}p < 0.01$, $^{**}p < 0.001$).

toxicity than that in the *wildtype* (*N2*). This may be due to the deletion sequences in *cyp35a2* mutant (*gk317*) and in *cyp35a2* RNAi were only partially overlapped, and thus, larger sequences of *cyp35a2* were deleted in *cyp35a2* RNAi in *cyp35a2* mutant (*gk317*) than in *cyp35a2* RNAi in *wildtype N2* (Supplementary Fig. 5).

Subsequently, the survival, growth, fertility, development and AChE activity were investigated on *cyp35a2* RNAi worms at the same exposure concentrations used for the wildtype *C. elegans*, which were 0.5, 5 and 50 μg L^{-1}. Fenitrothion exposure did not provoke any serious alteration on the 24 h-survival, growth or fertility in *cyp35a2* knocked-down worms, as was found in the *wildtype* worms (Supplementary Table 2).

FT-induced retardation and failure of development were significantly rescued in the *cyp35a2* RANi worms (Fig. 2B). When exposed to the highest FT level (50 μg L^{-1}), *wildtype C. elegans* could not complete their development and died 36 h after exposure. Conversely, the *cyp35a2* RNAi worms survived until the end of the experiment, and after 96 h of exposure, about 70% of the population had reached the L1–L3 stages.

Concentration-dependant inhibition of the AChE activity was observed in *wildtype C. elegans* exposed to FT. Even in the worms exposed to the lowest tested concentration of FT (0.5 μg L^{-1}), about 50% of the enzyme activity was inhibited. However, in *cyp35a2* RNAi worms, FT-induced inhibition of AChE activity was rescued, as no statistically significant inhibition of the AChE activity was observed at any FT concentration in *cyp35a2* RNAi worms (Fig. 4).

Overall data with *cyp35a2* RNAi worms indicated that development and AChE activity, which were the most seriously affected endpoints on FT exposure were significantly rescued in FT exposed *cyp35a2* RNAi *C. elegans*. These results suggest that the *cyp35a2* gene expression is required for inhibition of the AChE activity, and may have a negative effect on the worm's development. It is interesting to note that when *cyp32a2* is silenced, FT did not cause any effect on AChE activity (Fig. 4), but did cause some toxicity (Table 1). Given that OP metabolism *via* CYP is not fully understood in *C. elegans*, it cannot be ruled out that there may be other minor pathways that OP compounds exert toxicity independently from AChE inhibition. Indeed, FT altered not only expression of *cyp35a2* gene, but also, that of other group of genes such as, *hsp-16.2*, *vit-2*, and *ape-1* genes (Supplementary Fig. 3). The degree of alteration of these genes was not as significant as that of *cyp35a2* gene, however, this result may insinuate existence of other minor toxicity pathways of FT in *C. elegans* than CYP dependant AChE inhibition.

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Table 1
Estimation of 24 h EC50s of FT in *C. elegans*. The median effective concentration was estimated using immobility as an endpoint and derived through Probits analysis. Three replicates for each concentration was conducted.

Chemicals	Strains	24 h EC50s[a] (mg L^{-1})	95% Confidence interval (mg L^{-1})
FT	WT[b]	0.501	0.079 < EC50 < 1.374
	WT;*cyp35a2* RNAi[c]	0.991	0.354 < EC50 < 3.291
	cyp35a2(*gk317*)[d]	1.403	0.232 < EC50 < 4.923
	cyp35a2(*gk317*);*cyp35a2* RNAi[e]	3.275	0.884 < EC50 < 59.664

[a] EC50 means the median effective concentration (mg L^{-1}).
[b] WT means *wildtype* (N2).
[c] WT was fed with *cyp35a2* RNAi bacteria.
[d] *cyp35a2* Loss of function mutant strain.
[e] *cyp35a2*(*gk317*) Loss of function mutant was fed with *cyp35a2* RNAi bacteria.

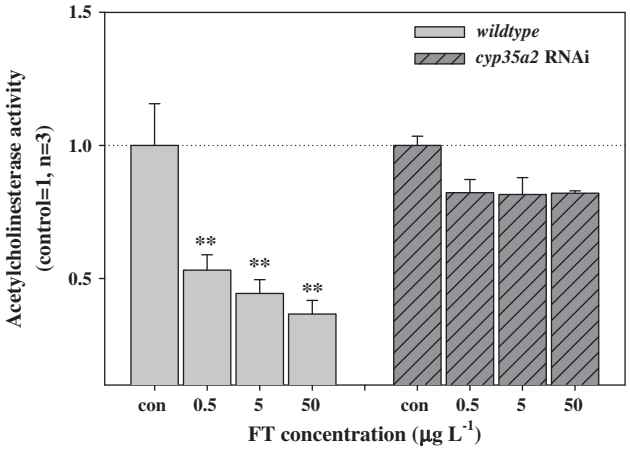

Fig. 4. Acetylcholinesterase activity in *wildtype* and *cyp35a2* RNAi *C. elegans* exposed to FT for 24 h. The results are presented as unit/mg protein (number = 5; mean ± standard error of the mean; *, significantly different from control, $^{*}p < 0.05$, $^{**}p < 0.01$).

Cytochrome P450 (CYP450s) plays critical roles in wide range of xenobiotic metabolism. Fenitrothion is known to be oxidized by CYP450 monooxygenases to the metabolite fenitrooxon in both animals and plants, which is a more powerful inhibitor of cholinesterase than its parent compounds (Ueyama et al., 2008; Abass et al., 2009). CYP450s are the principal phase I enzymes, which comprise a superfamily of heme-containing monooxygenases, with a wide array of substrate specificities (Usmani et al., 2004). There are known to be more than 2000 CYPs widely distributed throughout animals, plants and microorganisms. However, even though the entire genome of *C. elegans* was sequenced several years ago, little is known about xenobiotic metabolism in this organism (Lindblom and Dodd, 2006). There are at least 86 genes in the *C. elegans* genome that encode CYP proteins (Menzel et al., 2001) and one CYP, DAF, has been defined genetically and is well characterized as an important enzyme in the dauer pathway (Gill et al., 2004).

It would be important to identify how CYP pathway mediated toxicity observed in *C. elegans* study can be extrapolated to other species, as *C. elegans* is an important model system for human toxicity screening, as well as, an emerging model in ecotoxicology. However, there is still a serious lack of information on CYP function in toxic metabolism to draw any general conclusion about the correlation between the findings from *C. elegans* and other species. This is a challenging task, and may be achieved by accumulation of careful comparative toxicity studies. This aspect was recently stressed in the study by Leung et al. (2010), where they reported a lack of CYP1 family enzymes in *C. elegans* by comparing worms' response to aflatoxin B1, which is metabolized in mammals by CYP1, CYP2, and CYP3 family enzymes, and that to benzo[a]pyrene, which is metabolized in mammals by CYP1 family enzymes. They also reported sensitive response of *C. elegans* to OP compound, Chlorpyrifos, in DNA repair deficient strain (xpa-1), suggesting *in vivo* metabolic activation capacity of OP, probably *via* CYP2. As, it has been reported that nematodes have CYP2-like CYP14, CYP33, CYP34, and CYP35 families and in particular, *C. elegans* CYP35 genes are responsive to a variety of xenobiotic stressors including, PCBs (Menzel et al., 2001, 2005).

C. elegans cyp35a2 was reported to be involved in the fat storage pathway, however, the exact biological function of *cyp35a2* has still not been fully characterized. Therefore, our data suggests that *cyp35a2* may be involved in FT metabolism and/or toxicity in *C. elegans*. In our previous studies using metals (Cd and As; Roh et al., 2006), organic compound, (DEHP; Roh et al., 2007) and nanoparticles (CeO_2 and TiO_2, Roh et al., 2010), increased expressions of this gene were also observed. In our recent paper on nanoparticles, *cyp35a2* gene knock down was conducted to investigate the potential role of the *cyp35a2* gene in the physiological disturbance induced by NPs. Reduced fertility in wildtype *C. elegans* on nanoparticles exposure was rescued in *cyp35a2* RNAi worms, suggesting a potential role of the *cyp35a2* gene in nanoparticles induced toxicity. Screening of the LC50s of cadmium was also conducted using various potential stress response mutant strains, including *cyp35a2* (*gk317*), where defection of this gene was found to render worms slightly more tolerant to cadmium, suggesting involvement of this gene in cadmium toxicity. Taking the overall results of our studies, as well as those found in other investigations, it would appear that a wide range of chemicals, with different mode of actions, could induce *cyp35a2* gene expression in *C. elegans*.

4. Conclusion

In conclusion, FT exposure may provoke serious consequences on the *C. elegans* population, as it induced significant developmental disturbance. The increased expression of the *cyp35a2* gene on FT exposure occurred concomitantly with inhibition of AChE, development failure and immobility, suggesting involvement of this gene in FT toxicity. Functional analysis with *cyp35a2* RNAi worms indicated that the 24 h-EC50s of *C. elegans* on FT exposure were in the order of *cyp35a2* RNAi in *cyp35a2* mutant (*gk317*) > *cyp35a2* mutant (*gk317*) > *cyp35a2* RNAi in *wildtype* (*N2*) > *wildtype* (*N2*). The higher EC50 values of *cyp35a2* RNAi and *cyp35a2* mutant (*gk317*) compared to that of *wildtype C. elegans* strongly supported that *cyp35a2* gene plays an important role in the behavioral toxicity of FT towards *C. elegans*. Toxicity of FT on worms' immobility, development and AChE activity was also significantly rescued in *cyp35a2* RNAi *C. elegans*, suggesting that the *cyp35a2* gene expression is required for inhibition of the AChE activity, and may have a negative effect on the worm's behavior and development. The results also suggest that RNAi seems to be an ideal biomarker

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discovery tool in ecotoxicological research, as it can reveal the physiological meaning of altered gene expressions and thus, help identify the mechanism-based biomarkers for toxicity testing and risk assessment.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2011.05.010.

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