Effects of Perfluoro Organic Compound Toxicity on Nematode *Caenorhabditis elegans* Fecundity

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Toxicity studies using the nematode *Caenorhabditis elegans* (*C. elegans*) as a model organism have shown that perfluoro organic compounds have sub-lethal toxicity at the 10 pM–100 nM range on multi-generation assays. We examined the acute lethal toxicity and multi-generational sublethal toxicity (fecundity and reproduction) of perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA) and salts of perfluoro-1-octansulfonic acid (PFOS) using 1.7% agar Nematode Growth Medium (NGM) plates. The fluorine compounds affected the fecundity of *C. elegans* at concentrations 10^5- to 10^8-fold lower than the median effective concentrations (EC_{50}). In particular, worm abundance during the first generation did not differ significantly from controls, while in contrast, the number of worms in the fourth generation at 10 pM PFOS-tetraethylammonium (TEA) decreased significantly to about 50% of control ($p < 0.01$) and the number of eggs and worms in the fourth generation at 1 nM PFNA decreased significantly to about 30% of control ($p < 0.01$). However, no dose–response relationship was observed in this study. We confirmed that perfluoro organic compounds strongly disrupt fecundity in *C. elegans*.

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INTRODUCTION

Perfluoro organic compounds are widely used in a variety of housekeeping products due to their water repellent properties, and these synthetic chemicals have been detected in wild organisms.[1,2] Recently, many ecotoxicologists have focused their attention on the toxicity of low-dose and long-term exposure to perfluoro organic compounds, but due to their poor solubility in water, little information is available. Poor solubility in water makes it difficult to conduct laboratory-scale bioassay testing using fish, the most popular test organism.

The soil dwelling nematode, *Caenorhabditis elegans* (*C. elegans*), has been shown to be a suitable test organism, showing both lethal and sublethal toxicity endpoints, in the ecotoxicological assessments of liquid and soil media.[3–9] Previous studies demonstrated that the use of 1.7% agar Nematode Growth Medium (NGM) plates and *C. elegans* permits multi-generation sublethal toxicity testing of chemicals that are poorly soluble in water.[10] In this study, we investigated the lethal and sublethal effects of some kinds of perfluoro organic compounds on *C. elegans*.

MATERIALS AND METHODS

Chemicals —— Agar, cholesterol, dimethyl sulfoxide (DMSO), perfluoro-1-octansulfonic acid (PFOS)-K and sodium chloride used in this study were purchased from Wako Pure Chem (Osaka, Japan). Reagent grade bacto yeast extract and bacto tryptone was supplied by BECKTON-DICKINSON (MD, U.S.A.). perfluorooctanoic acid (PFOA) and perfluorononanoic acid (PFNA) were obtained from TOKYO Kasei (Tokyo, Japan). PFOS-tetraethylammonium (TEA) was supplied by Aldrich (Tokyo, Japan).

Animals and Culture Conditions —— Wild-type N2 strain *C. elegans* were used in this study. Worms were maintained and handled basically as previously described.[11] Briefly, five to ten worms were fed *Escherichia coli* (*E. coli*) DH5α spread onto NGM agar plates containing 0.5 μM cholesterol at 16°C, and subculturing was performed at 5- to 6-day in-

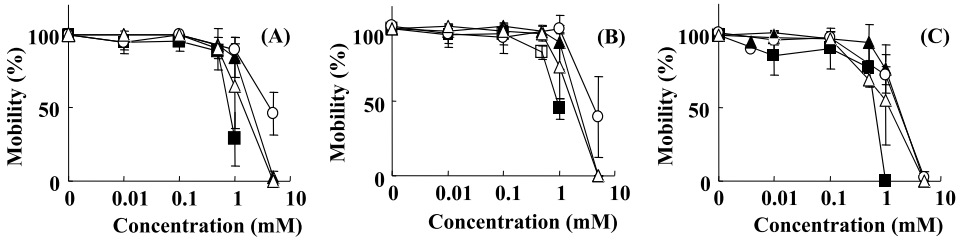

Fig. 1. Acute Lethal Toxicity of Fluorine Compounds on *C. elegans*

(A) *C. elegans* mobility at 4 hr, (B) *C. elegans* mobility at 24 hr, (C) *C. elegans* mobility at 48 hr. ▲, PFOA; ■, PFNA; ○, PFOS-TEA; △, PFOS-K. Each value represents the mean ± SEM of 9 experiments.

tervals.

Synchronized Cultures of *C. elegans* —— For toxicity assays, worms were washed out from the NGM agar plates using S-basal medium, washed with S-basal medium twice, and then synchronized according to the alkali-bleaching method. Adult worms were dissolved in 12% sodium hypochlorite containing 0.5 M potassium hydroxide, and the eggs were collected by centrifugation. Then, an appropriate number of eggs were suspended in S-basal medium without cholesterol and incubated at 20°C for 20 hr with shaking. Over 90% of eggs hatched, and *C. elegans* larvae were synchronized at the L1 larval stage. These larval cultures were used for the acute toxicity assay.

Exposure to Chemicals —— DMSO was used as a toxicant vehicle at a final concentration of 0.5% in this study.[12] The DMSO solutions of test chemicals were added to sterilized NGM 1.7% agar medium containing 0.5 μM cholesterol. After the mixture was sonicated, the medium was poured into 6-cm petri plates for exposure testing. A suspension of L1 larval stage worms, produced by the alkali-bleach age-synchronization method, was poured onto the plates and incubated at 16°C.

Acute Toxicity Test —— About 20 L1 larvae were poured onto the each plate containing 0, 0.01, 0.1, 0.5, 1.0 and 5.0 mM of chemicals. The total number of worms and the number of active worms on each plate were counted under a dissecting microscope at 1, 2, 3, 4, 24, and 48 hr after staring exposure to estimate mobility. The mobility was expressed with the percentages of number of moving worms by total number of worms in the treatment. All experiments were performed at 16°C without food in triplicate and repeated at least three times on different occasions. The median effective concentrations (EC_{50}), calculated using the PROBIT method.

Multi-Generation Test —— *E. coli* DH5α was spreded on the plates before multi-generation test. Then first generation L1 larvae were poured onto the each plate to start the test. When worms grew to L4 larvae, one worm was picked up and transferred to a new plate of the same composition, and incubated at 16°C. The number of worms and eggs on the plates were counted under a dissecting microscope at 24-hr intervals. The day when the first offspring was identified was defined as the first day. The second-generation worms were allowed to grow to L4 larvae stage on the same plate, and then one worm was picked up and transferred to a new plate of the same composition. These steps were repeated until the fifth generation was cultured. These experiments were carried out in triplicate and on at least three times on different occasions.

Statistical Analyses —— All results were expressed as mean ± SEM. Analysis of variance (ANOVA) was performed to identify the significantly difference from the control group. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

The results of the acute toxicity test are shown in Fig. 1. None of control worms died during the observation. All chemicals used in this study at concentrations up to 0.1 mM show no acute lethal toxicity against *C. elegans* until incubation of 48 hr [Fig. 1(C)]. Acute lethal toxicity appeared at concentrations greater than 0.5 mM and did not depend on the incubation time [Fig. 1(A)–(C)]. The EC_{50}, calculated using the PROBIT method, are shown in Table 1. According to acute toxicity tests using *C. elegans*, perfluoro organic compounds have EC_{50} values less than 5 mM, weaker toxicity than shown by exposure to nonylphenol and octylphenol.[13]

Table 1. EC_{50} Values at Exposure Time of Fluorine Compounds

Exposure time (hr)	EC_{50} (mM)			
	PFOA	PFNA	PFOS-TEA	PFOS-K
1	3.85	1.00	—	3.35
2	2.80	0.90	—	2.30
3	2.70	0.95	—	2.30
4	2.65	0.82	4.70	1.90
24	2.75	0.92	4.30	2.25
48	2.35	0.66	2.22	1.35

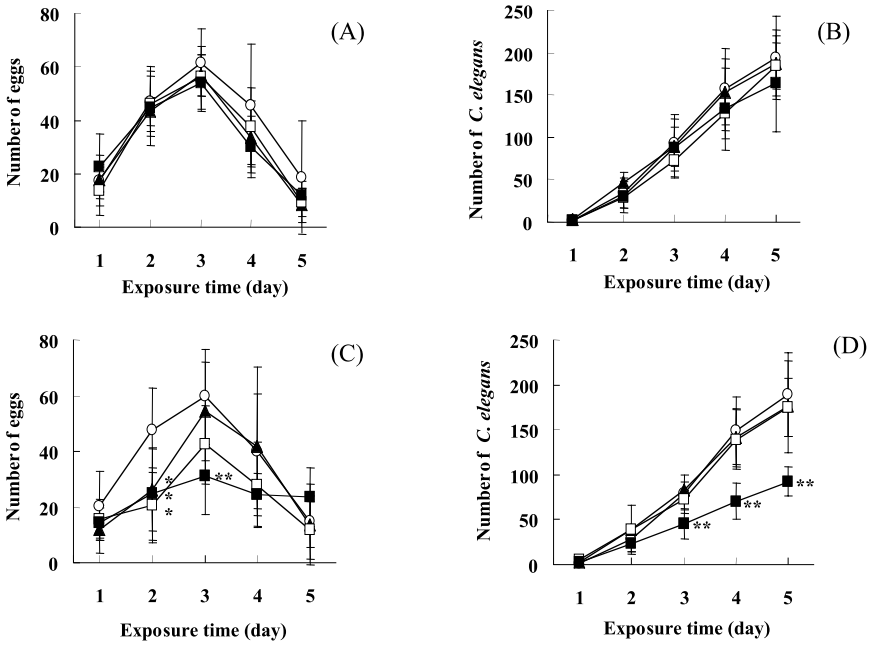

Fig. 2. Effect of PFOA on the Fecundity of *C. elegans*

(A) Egg number at the 1st generation, (B) *C. elegans* number at the 1st generation, (C) Egg number at the 4th generation, (D) *C. elegans* number at the 4th generation. ○, Control; ▲, 100 pM; □, 1 nM; ■, 10 nM. Each value represents the mean ± SEM. *Significantly difference from the control group at $p < 0.05$ by ANOVA. **Significantly difference from the control group at $p < 0.01$ by ANOVA.

The results of multi-generation test of low concentration perfluoro organic chemicals are shown in Figs. 2–5. In this assay, the CV ranged from 25% to 50% within assays and from 10% to 40% between assays. At concentrations about 100-fold lower than the EC_{50} (100 μM), the worm abundance from the first generation to the second generation showed a decrease so extreme that sub-culturing was discontinued. In the fourth generation, chemicals at concentrations less than 10 nM were shown to affect the fecundity of *C. elegans*. Worm abundances and egg numbers during the first generation did not differ significantly from the controls for all chemicals in use this study [Figs. 2–4(B)]. However, in the fourth generation at 1 nM PFNA, the number of eggs and worms decreased significantly to about 30% of the control ($p < 0.01$), as shown in Fig. 3(B) and (C), respectively. This concentration is $1/10^6$ of the 24 hr-EC_{50} value (0.92 mM). Generation-response and concentration-response relationships were not observed in PFOA and PFNA, and the effects were almost the same in the second, third, fourth, and fifth generations. Sulfonic acid derivatives, PFOS-TEA and PFOS-K, affected the fecundity rate of *C. elegans* at lower concentrations, those similar to the concentrations of organotin compounds know to cause the development of imposex in the rock shell, *Thais clavigera*.[14] In particular, 10 pM PFOS-TEA, an exposure concentration 5×10^8-fold lower than the 48 hr-EC_{50} (2.22 mM), produced a significant decrease (about 50% of the control) in the number of worms in the fourth generation ($p < 0.01$) (Fig. 4).

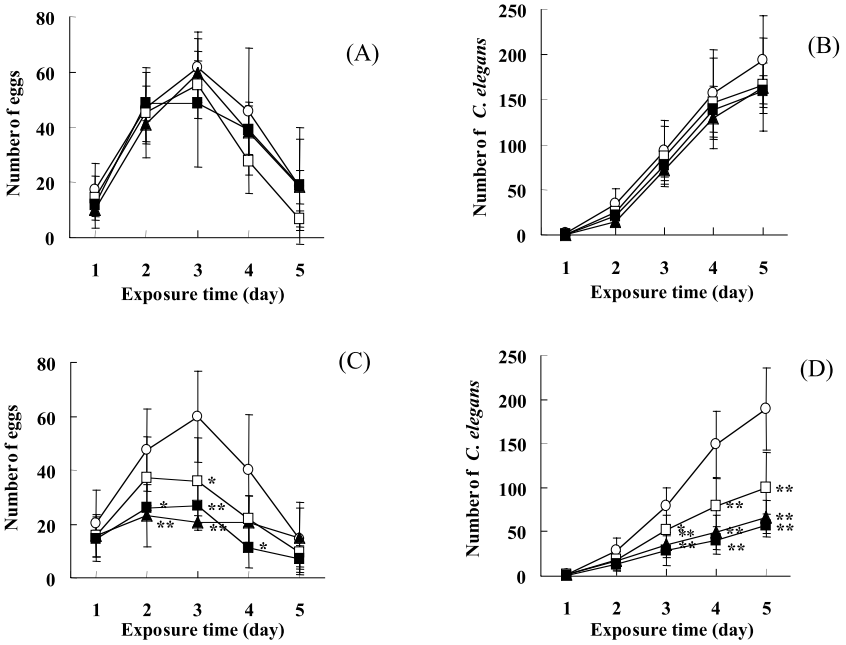

Fig. 3. Effect of PFNA on the Fecundity of *C. elegans*

(A) Egg number at the 1st generation, (B) *C. elegans* number at the 1st generation, (C) Egg number at the 4th generation, (D) *C. elegans* number at the 4th generation. ○, Control; ▲, 1 nM; □, 10 nM; ■, 100 nM. Each value represents the mean ± SEM. *Significantly difference from the control group at $p < 0.05$ by ANOVA. **Significantly difference from the control group at $p < 0.01$ by ANOVA.

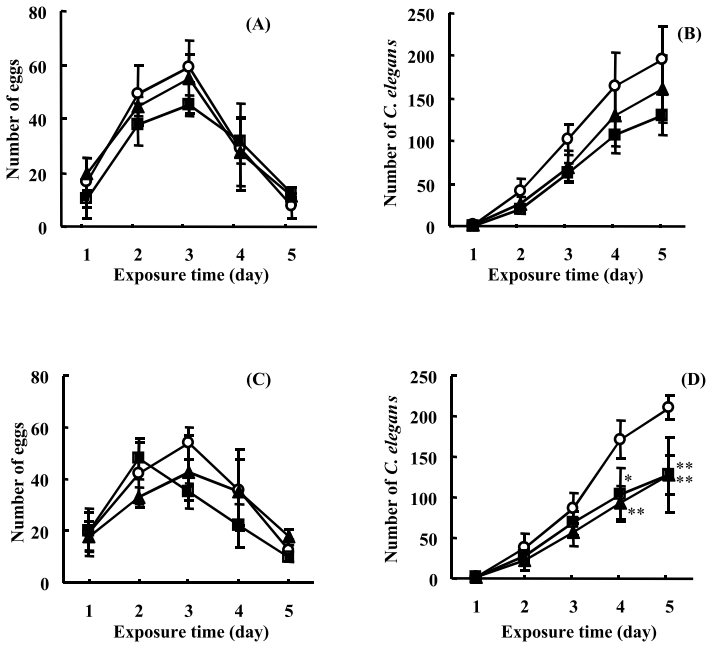

Fig. 4. Effect of PFOS-TEA on the Fecundity of *C. elegans*

(A) Egg number at the 1st generation, (B) *C. elegans* number at the 1st generation, (C) Egg number at the 4th generation, (D) *C. elegans* number at the 4th generation. ○, Control; ▲, 10 pM; ■, 100 pM. Each value represents the mean ± SEM. *Significantly difference from the control group at $p < 0.05$ by ANOVA. **Significantly difference from the control group at $p < 0.01$ by ANOVA.

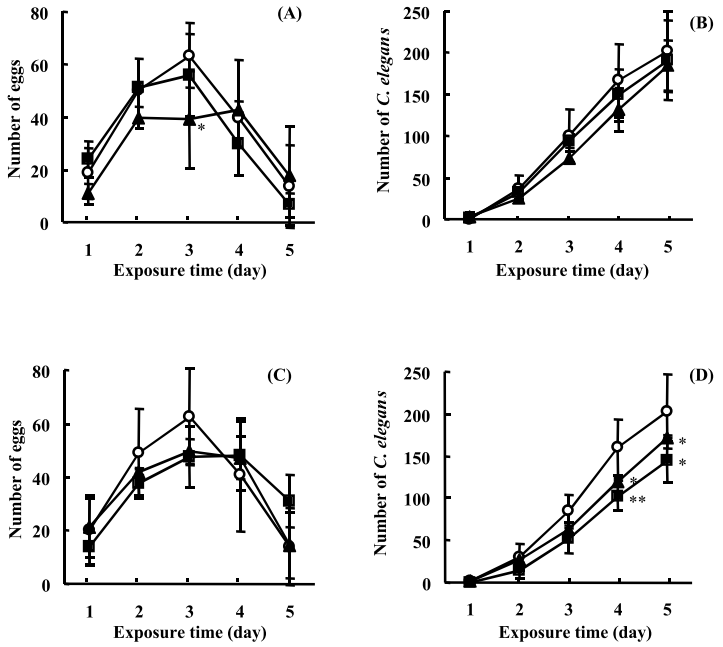

Fig. 5. Effect of PFOS-K on the Fecundity of *C. elegans*

(A) Egg number at the 1st generation, (B) *C. elegans* number at the 1st generation, (C) Egg number at the 4th generation, (D) *C. elegans* number at the 4th generation. ○, Control; ▲, 1 nM; ■, 100 nM. Each value represents the mean ± SEM. *Significantly difference from the control group at $p < 0.05$ by ANOVA. **Significantly difference from the control group at $p < 0.01$ by ANOVA.

Since these perfluoro organic compounds are poor soluble compounds in water, a dose–dependent response was also not observed in this case. The ranking of sublethal toxicity for these compounds was PFOS-TEA > PFOS-K > PFOA ≒ PFNA. Further examination is necessary to elucidate the structure-activity relationship of these chemicals.

Studies examining the uptake and accumulation of these chemicals will be necessary to clarify whether perfluoro organic compounds can alter fecundity and reproduction of the nematode *C. elegans* even at quite low concentrations, as shown in this study. Nematode biomass has been decreasing in the presence of perfluoro organic compound pollution. Sanderson et al have reported effects of PFOA and PFOS on the zooplankton community.[15] Since nematodes and zooplankton species play essential roles in food chains, these results may be ecologically relevant for the protection of ecosystems.

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