Using the Nematode *Caenorhabditis elegans daf-16* Mutant to Assess the Lifespan Toxicity of Prolonged Exposure to Ecotoxic Agents

Masaru Kurauchi, Hisashi Morise, and Toshihiko Eki*

Division of Life Science and Biotechnology, Department of Ecological Engineering, Toyohashi University of Technology, 1–1 Hibarigaoka, Tempaku-cho, Toyohashi, Aichi 441–8580, Japan

(Received November 28, 2008; Accepted May 25, 2009; Published online June 15, 2009)

Nematodes are highly abundant organisms found in soil or sedimentary habitats. The free-living nematode *Caenorhabditis elegans* (*C. elegans*) has been used as an excellent model for monitoring ecotoxicity in soil. We have previously demonstrated that the lifespan of *C. elegans* can be used as an endpoint for detecting the ecotoxicity of heavy metals and detergents, and have developed a novel ecotoxicity assay based on their shortened lifespan. Herein we used a *daf-16*(*mu86*) mutant CF1038 strain, which has a deficient transcription factor DAF-16 regulating a variety of genes involved in longevity and stress response, for ecotoxicity assays. We carefully examined the effects on reproduction, larval growth, and lifespan in the short-lived CF1038 strain and wild-type N2 strain in the presence of a perfluoro organic compound (pentadecafluorooctanoic acid) and an organophosphate insecticide (dichlorvos) in addition to heavy metals ($CuSO_4$ and $CdCl_2$) and detergents (sodium dodecyl sulfate and a commercially available household detergent). Unexpectedly, both strains exhibited comparable reductions in these endpoints including lifespan by exposure to these ecotoxicants, indicating that DAF-16 does not largely contribute to tolerance to these agents. By virtue of a shorter assay period, the lifespan-based assay using the *daf-16* mutant can be useful for assessing the ecotoxicity of a variety of ecotoxic chemicals.

*To whom correspondence should be addressed: Division of Life Science and Biotechnology, Department of Ecological Engineering, Toyohashi University of Technology, 1–1 Hibarigaoka, Tempaku-cho, Toyohashi, Aichi 441–8580, Japan. Tel.: +81-532-44-6907; Fax: +81-532-44-6929; E-mail: eki@eco.tut.ac.jp

Key words —— ecotoxicity test, lifespan assay, *Caenorhabditis elegans*, *daf-16* mutant, ecotoxic compounds

INTRODUCTION

Nematodes are highly abundant organisms found in soil or sedimentary habitats, and free-living nematode species play important roles in decomposition and nutrient cycling.[1] Relative to their significance in the soil ecosystem, nematodes are suitable model organisms for monitoring soil conditions and ecotoxicity.[2] The free-living nematode *Caenorhabditis elegans* (*C. elegans*) has been widely used as an excellent model for studying the fundamental processes of multicellular eukaryotes and can be easily maintained and manipulated in the laboratory. By virtue of these properties, several toxicity tests using *C. elegans* have been developed for ecological risk assessment.[3,4] These tests were effective in the detection of contamination of ecotoxic agents by monitoring the toxic effects on mortality, reproduction, larval growth, and so on as ecologically relevant endpoints. Thus several endpoints have been used for acute toxicity testing with *C. elegans* and the effects are assessed in relatively short-term treatments ($\leq$ 3 days). Since living organisms in polluted environments are continuously exposed to toxicants, for example by intake of foods and fluids, and are chronically damaged, we examined the long-term influence on the lifespan of the nematode *C. elegans* and observed the concentration-dependent shortening of the lifespans of nematodes by prolonged exposure to heavy metals and detergents.[5] Our results indicate that the nematode lifespan is a useful endpoint for assessing ecotoxicity of a variety of agents.

In this study, we examined the effects on the *daf-16*(*mu86*) mutant without functional DAF-16 protein[6] resulting from exposure to ecotoxic agents. The transcription factor DAF-16[7] is known to regulate multiple biological processes including lifespan, stress response, dauer formation, and metabolism in *C. elegans*.[8] Since DAF-16 regulates many genes involved in cellular stress response,[9] the *daf-16* mutant might be more sensitive to a variety of toxicants than the wild-type nematode. In addition, DAF-16 influences the rate of aging in response to insulin/insulin-like growth factor 1 (IGF-1) signaling and *daf-16* null mutants have shortened lifespans.[10] Use of the *daf-16* mutant may allow us

to minimize the assay period required for lifespan measurement. We previously examined the toxic effects on reproductive capacity, larval growth rate, and lifespan in both the *daf-16* and wild-type nematodes by exposure to heavy metals (copper and cadmium) and detergents [sodium dodecyl sulfate (SDS; Sigma, St. Louis, MO, U.S.A., L-4390) and a commercially available surfactant]. Furthermore, we recently studied toxicity caused by exposure to two kinds of ecotoxicants, a perfluoro organic compound (perfluorooctanoic acid) and an organophosphate insecticide (dichlorvos). In these experiments, both strains were comparably affected by the toxicants tested in a concentration-dependent manner, suggesting that the *daf-16* mutant can be used for a lifespan assay. Because a long culture period (> 1 month) is a serious problem in an original lifespan assay using wild-type nematodes, the improved method using the short-lived *daf-16* mutant is practically useful for assessment of ecotoxicity resulting from prolonged exposure to hazardous agents by virtue of its short assay period.

MATERIALS AND METHODS

Chemicals Tested —— The following ecotoxic agents were used for the experiments: $CuSO_4 \cdot 5\,H_2O$ (Wako Pure Chemical Industries, Osaka, Japan, No. 039-04412), $CdCl_2 \cdot 2.5\,H_2O$ (Wako Pure Chemical Industries, No. 038-00102), SDS, a commercially available household detergent (33% [v/v] concentration of detergent mixture of alkyl ether sulfate ester, alkyl amine oxide, alkyl glycoside, and alkyl hydroxysulfobetaine), pentadecafluorooctanoic acid (PFOA; Tokyo Chemical Industry Co., Tokyo, Japan, P0764), and 2,2-dichlorovinyl dimethyl phosphate (dichlorvos; Hayashi Pure Chemical Inc., Osaka, Japan, No. 45441).

Preparation of Nematode Growth Medium (NGM) Plates for Toxicity Test —— *C. elegans* wild-type strain Bristol N2 and the *daf-16*(*mu86*) mutant strain CF1038[6] were provided by Dr. I. Katsura (National Institute of Genetics, Mishima, Japan) and the *Caenorhabditis* Genetics Center (Minneapolis, MN, U.S.A.), respectively. Nematodes were maintained at 20°C on agar plates of NGM supplemented with *Escherichia coli* strain OP50.[11] The NGM plates containing toxicants (*i.e.*, a test NGM plate) were prepared as described previously.[5] The NGM plate containing 0.5% (v/v) DMSO was used as control without toxicants in the experiments for PFOA and dichlorvos.

Determination of Body Length and Reproductive Capacity —— Growth and development of newly hatched nematodes on a test NGM plate was monitored by body length determination as described in our previous paper.[5] Reproductive capacity was monitored by determining the mean number of hatched progeny/nematode (*i.e.*, brood size) in the entire brood period as described previously[5] and the measurement was carried out using > 6 animals/group, at least in duplicate.

Determination of Lifespan (Lifespan Assay) —— For the determination of lifespan, > 30 animals (N2 or CF1038 strain) raised to the L4 stage were transferred into four fresh NGM plates containing 25 μM 5-fluoro-2′-deoxyuridine (Sigma, F-0503) with or without toxicant, and grown at 20°C. In brief, after placement on test plates containing the indicated concentration of toxicants (lifespan = 0), the viability of the adult animals was checked under a stereomicroscope every day. When the nematodes stopped moving, they were checked for viability by tapping the plates and by gently touching them with a platinum picker as described previously.[5] The experiments were independently repeated at least twice.

Statistical Analysis —— The software package JMP 6.0.3 (SAS Institute Inc., Cary, NC, U.S.A.) was used for statistical analyses for lifespans and growth rates as described previously.[5]

RESULTS

Toxic Effects on Reproduction and Growth of Wild-type and *daf-16* Mutant Nematodes

In this study, we examined the biological effects in two nematode strains, the wild-type N2 and the *daf-16*(*mu86*) mutant CF1038, resulting from exposure to toxic agents by conventional agar plate-based assays as described previously.[5] First, we tested the reproductive capacity of parental animals exposed to various concentrations of heavy metal ($CdCl_2$), detergent (SDS), and two ecotoxic chemicals (PFOA and dichlorvos) (Table 1). The tested animals were maintained with or without toxicants over the entire brood period to determine the mean number of hatched progeny/animal (*i.e.*, brood size). The number of progeny produced by

Table 1. Influence on Reproduction of *C. elegans* by Exposure to Four Ecotoxicants

Toxicant tested	No. progeny/animal ± S.D.	
	N2 strain (wild-type)	CF1038 strain (*daf-16* mutant)
$CdCl_2$ (mM) 0	190.3 ± 24.5	224.1 ± 1.2
0.03	156.5 ± 8.7	181.1 ± 7.8
0.1	164.5 ± 18.6	161.3 ± 19.7
0.3	38.2 ± 11.7	35.2 ± 1.2
SDS (%) 0	210.9 ± 31.8	192.7 ± 66.8
0.001	203.1 ± 41.1	183.6 ± 88.5
0.003	154.3 ± 67.0	167.1 ± 73.8
0.006	62.4 ± 37.4	92.1 ± 47.5
PFOA (mM) 0 (0.5% DMSO; control)	159.0 ± 5.9	181.8 ± 21.4
0.3	130.8 ± 5.8	173.1 ± 9.2
1.0	92.5 ± 2.6	98.8 ± 4.0
2.0	26.3 ± 2.0	37.1 ± 2.2
Dichlorvos (μM) 0 (0.5% DMSO; control)	172.5 ± 14.3	213.2 ± 17.2
9.4	164.8 ± 15.3	206.3 ± 9.9
31	163.1 ± 18.1	153.0 ± 33.0
94	135.7 ± 13.6	137.1 ± 17.2

No. hatched progeny from the parent nematodes cultured under the indicated conditions was counted as described in Materials and Methods.

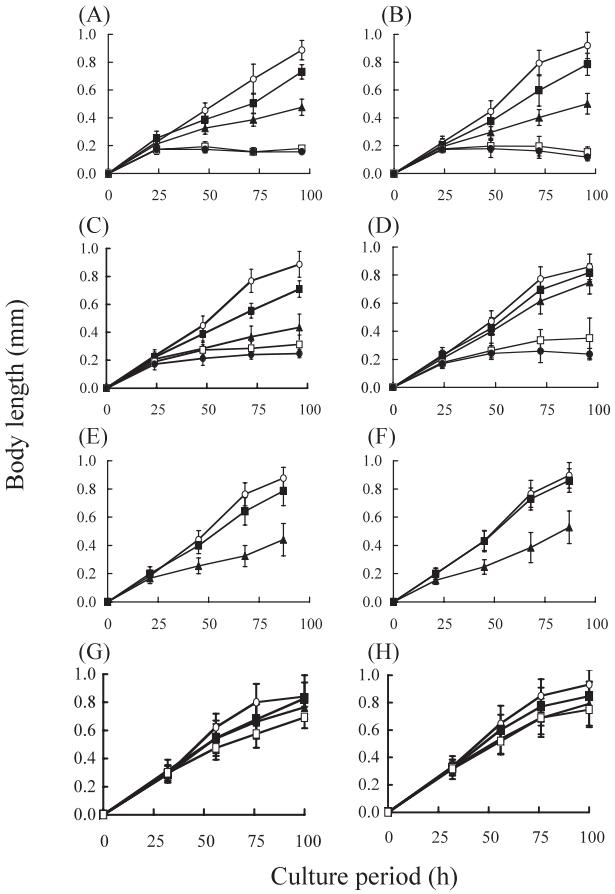

Fig. 1. Larval Growth of Nematodes Exposed to Four Ecotoxic Agent

Fertilized eggs from wild-type N2 (A, C, E, and G) or *daf-16* mutant CF1038 (B, D, F, and H) strain were hatched and grown on test NGM agar plates in the absence (control, open circle) or presence of 0.03 (closed square), 0.1 (closed triangle), 0.3 (open square), and 1.0 mM (closed circle) of $CdCl_2$ (A and B), 0.001% (closed square), 0.003% (closed triangle), 0.006% (open square), and 0.01% (closed circle) of SDS (C and D), 0.3 (closed square) and 1.0 mM (closed triangle) of PFOA (E and F), and 9.4 (closed square), 31 (closed triangle), and 94 μM (open square) of dichlorvos (G and H). The body lengths of worms (n = 30 per point) were determined to monitor growth. The mean body lengths with standard deviations are plotted. The growth rates in each group were estimated by fitting a growth curve as follows: (A) control (N2 strain), 0.23; 0.03 mM $CdCl_2$, 0.15; 0.1 mM, 0.05; 0.3 mM, 0 (growth arrest); 1.0 mM, 0 mm/24 h; (B) control (CF1038 strain), 0.26; 0.03 mM $CdCl_2$, 0.20; 0.1 mM, 0.09; 0.3 mM, 0; and 1.0 mM, 0 mm/24 h; (C) control (N2 strain), 0.25; 0.001% SDS, 0.18; 0.003%, 0.10; 0.006%, 0.05; 0.01%, 0 mm/24 h; (D) control (CF1038 strain), 0.26; 0.001% SDS, 0.22; 0.003%, 0.20; 0.006%, 0.06; 0.01%, 0 mm/24 h; (E) control (0.5% DMSO) (N2 strain), 0.26; 0.3 mM PFOA, 0.23; 1.0 mM, 0.11 mm/24 h; (F) control (CF1038 strain), 0.26; 0.3 mM PFOA, 0.25; 1.0 mM, 0.14 mm/24 h; (G) control (0.5% DMSO) (N2 strain), 0.26; 9.4 μM dichlorvos, 0.24; 31 μM, 0.21; 94 μM, 0.16 mm/24 h; (H) control (CF1038 strain), 0.28; 9.4 μM dichlorvos, 0.25; 31 μM, 0.20; 94 μM, 0.20 mm/24 h, respectively.

N2 animals with 0.3 mM $CdCl_2$ or 0.006% SDS was reduced to 20% and 30% of that of the corresponding control animals, respectively (Table 1), as observed previously.[5] The mean brood size of the *daf-16*(*mu86*) animals without toxicants is 203.0, which is slightly higher relative to that of the wild-type animals (183.2), as observed in an another *daf-16* mutant [*daf-16*(*m*26)] by others.[12] In the presence of $CdCl_2$ and SDS, concentration-dependent reductions in reproductive capacity in the *daf-16* animals were comparable to those found in the N2 animals treated with the same agents. The brood size of N2 and CF1038 animals treated with > 1.0 mM PFOA was significantly decreased. On the other hand, the reproduction capacity of each strain was not largely affected by dichlorvos (Table 1).

We next examined the growth and development of nematode larvae in the presence or absence of the agents. Growth of hatched larvae was monitored by measurement of the body lengths of the nematodes (N2 and CF1038 strains) treated with various concentrations of the agents. The growth curve from the mean body length in each group is shown in Fig. 1. For example, the growth curves from N2 and CF1038 animals treated with various concentrations of $CdCl_2$ are shown in Fig. 1A and B, respectively. The growth rate estimated by fitting each growth curve from N2 and CF1038 control animals without $CdCl_2$ is 0.23 and 0.26 mm/24 h, respectively. The larval growth of N2 (Fig. 1A, C, E, and G) and CF1038 (Fig. 1B, D, F, and H)

animals was clearly and significantly decreased in the presence of $CdCl_2$ (Fig. 1A and B) and SDS (Fig. 1C and D), as reported previously.[5] Similar inhibition of larval growth was observed in the cultures with PFOA (Fig. 1E and F). For example, the mean body lengths of N2 and CF1038 animals treated with 1.0 mM PFOA was 0.25 ± 0.01 and 0.25 ± 0.01 mm for 48 hr, which is significantly smaller than that of control worms (0.44 ± 0.01 mm for the N2 strain; 0.43 ± 0.01 mm for the CF1038 strain) (both $p < 0.0001$). Growth of larvae for both strains was not inhibited with 0.5% DMSO that was commonly contained in the test NGM plates for PFOA (*i.e.*, 0.26 and 0.23 mm/24 h of the growth rate for N2; 0.26 and 0.26 mm/24 h for CF1038 animals with or without 0.5% DMSO). Unlike the other three toxicants, inhibition of larval growth with dichlorvos was not significant in both strains; for instance, < 40% of reduction in the larval growth rate was observed even in the presence of 94 μM dichlorvos (Fig. 1G and H). The inhibition of growth in nematodes is dependent on the concentration of cadmium, SDS, or PFOA. The median effective concentrations (EC_{50}s) were estimated as the concentration where the growth rate of the worms is decreased to 50% of that of the control (Fig. 1); EC_{50} values for the N2 and CF1038 animals with the various additives were approximately 0.04 and 0.06 mM cadmium, 0.002% and 0.004% SDS, 0.9 and 1.0 mM PFOA, respectively, indicating that these toxicants inhibit larval growth of both strains in a similar concentration range.

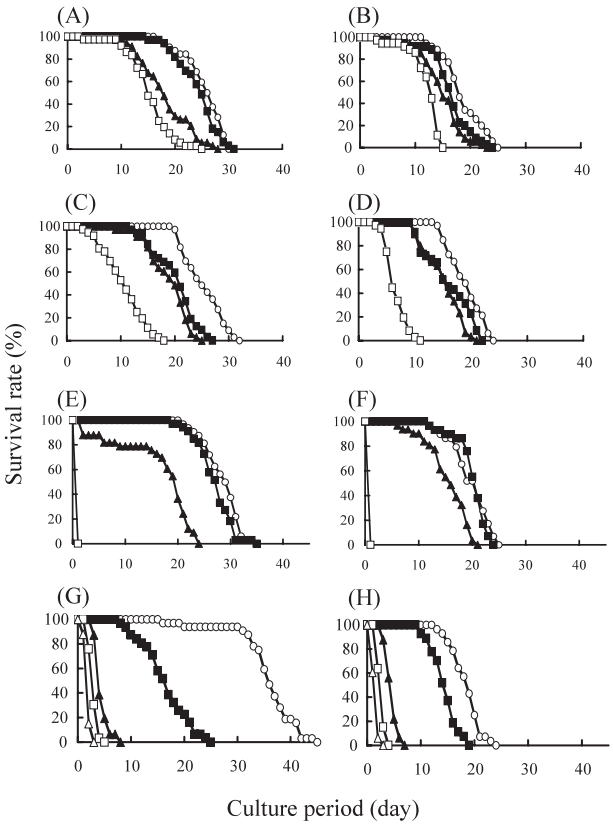

Fig. 2. Lifespans of Wild-type and *daf-16* Mutant Animals Exposed to Heavy Metals and Detergents

The wild-type N2 (A, C, E, and G) or *daf-16* mutant CF1038 (B, D, F, and H) animals were maintained in the absence (control, open circle) or presence of 0.1 (closed square), 0.3 (closed triangle), and 1.0 mM (open square) of $CuSO_4$ (A and B), 0.03 (closed square), 0.1 (closed triangle), and 0.3 mM (open square) of $CdCl_2$ (C and D), 0.001% (closed square), 0.003% (closed triangle), and 0.01% (open square) of SDS (E and F) and 0.0033% (closed square), 0.01% (closed triangle), 0.033% (open square), and 0.1% (open triangle) of a household detergent (G and H) to determine their lifespans.

Shortened Lifespans Resulting from Prolonged Exposure to Ecotoxicants in Wild-type and *daf-16* Mutant Animals

We assessed the long-term effects on lifespans of wild-type and *daf-16* animals continuously exposed to six toxicants. The survival fraction of adult nematodes maintained with different concentrations of toxicants was monitored. The resultant survival curves for heavy metals ($CuSO_4$ and $CdCl_2$), detergents (SDS and a household surfactant), PFOA, and dichlorvos are shown in Figs. 2 and 3. The mean lifespan of nematodes in each group with standard errors is summarized in Table 2. The mean lifespans in the wild-type N2 and *daf-16* mutant CF1038 animals maintained without toxicants in six experiments (Table 2) were 28.0 and 19.2 days, respectively. The *daf-16* mutant CF1038 strain had a shortened lifespan (*i.e.*, approximately 70% of the lifespan relative to that of the wild-type strain). The mean lifespan of nematodes was not significantly affected in the absence or presence of 0.5% DMSO in the test NGM plates for PFOA and dichlorvos (28.8 versus 26.3 days in N2 strain; 19.3 versus 19.0 days in CF1038 strain, respectively). The higher concentrations of toxicants caused acute toxicity and killed adult animals within a few days of transfer to the test plates; for example, those that contained 0.01% SDS, 0.1% of the detergent, or 310 μM dichlorvos (Figs. 2 and 3). Although some differences in toxic effects from a household detergent (Fig. 2G and H) and PFOA (Fig. 3A and B) were observed between N2 and CF1038 animals, both strains exhibited similar reductions in their lifespan by the other agents tested. In the assay for dichlorvos, weakly enhanced lethality of the N2 animals (or weak tolerance of the *daf-16* animals)

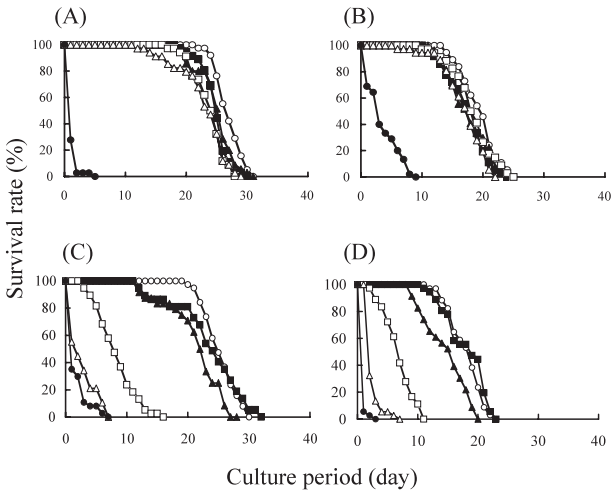

Fig. 3. Lifespans of Wild-type and *daf-16* Mutant Animals Exposed to PFOA and Dichlorvos

Lifespans of wild-type N2 (A and C) or *daf-16* mutant CF1038 (B and D) animals were determined in the absence (0.5% DMSO as control, open circle) or presence of 0.03 (closed square), 0.1 (closed triangle), 0.3 (open square), 1.0 (open triangle), and 3.0 mM (closed circle) of PFOA (A and B) and 3.1 (closed square), 9.4 (closed triangle), 31 (open square), 94 (open triangle), and 310 μM (closed circle) of dichlorvos (C and D).

was detected in the presence of 3.1 μM dichlorvos (Fig. 3C and D). The shortening of lifespans by exposure to the agents was clearly dependent on their concentrations. The EC_{50}s of the toxicants for N2 strain and CF1038 strain were respectively: 0.25 and 0.24 mM for $CdCl_2$; 0.004 and 0.0055% for SDS; 0.003 and 0.0055% for a household detergent; 2.0 and 2.1 mM for PFOA; and 17 and 21 μM for dichlorvos. The EC_{50} values for cadmium, SDS, and PFOA in both strains were essentially similar to those for short-term toxicity such as growth retardation of offspring. Finally, it is of note that use of the *daf-16* mutant allowed us to save 7–10 days in the assay period for measurement of lifespan in comparison with our previous method using a wild-type nematode (Figs. 2 and 3).

DISCUSSION

In this study, we examined the effects resulting from exposure to ecotoxic agents in *daf-16*(*mu86*) mutant[6] and wild-type nematodes. Several studies have revealed a close relationship between stress tolerance and longevity in *C. elegans*.[13] For example, the long-lived *daf-2* or *age-1* mutants exhibit increased resistance to heavy metals,[14] H_2O_2,[15] paraquat,[16] UV irradiation,[12] and a neurotoxicant,[17] indicating that both genes function negatively for lifespan and stress resistance. The *daf-2* and *age-1* genes encode insulin/IGF-1 receptor and the phosphoinositide 3-kinase catalytic subunit for *C. elegans* insulin/IGF-1 signaling pathway, respectively.[8] A forkhead transcription factor DAF-16[7] influences the rate of aging in response to insulin/IGF-1 signaling and regulates cellular stress response genes.[9] Therefore genetic mutations of the *daf-16* gene or DAF-16-regulated stress response genes likely cause a shortened lifespan as well as dysfunctions of tolerance to environmental stresses, and a lifespan assay using the *daf-16* mutant was assumed to be more sensitive to toxic agents than our previous method using a wild-type nematode[5] in a short period. So far, a few short-lived mutants (*e.g.*, *mev-1*, *gas-1*, *scl-1*, *daf-18*, and *daf-16*) have been reported. However, both *mev-1* and *gas-1* mutants are mainly sensitive to oxidative stresses.[18, 19] The *daf-18* gene acts in insulin/IGF-1 signaling pathway[20] and the *scl-1* gene is regulated by DAF-16.[21] Thus *daf-16* mutants are more suitable for a lifespan assay due to their short lifespan and possible enhanced sensitivity to toxicants. In our lifespan assay, the *daf-16*(*mu86*) mutant CF1038 strain exhibited a shortened lifespan as we expected and this result is consistent with previous reports by others[10] and our observations in the lifespan assay using *daf-16* RNAi-treated N2 nematodes.[22] Use of the *daf-16* mutant successfully allowed us to complete a lifespan assay in a shorter period rather using the previous method with a wild-type nematode as summarized in Table 2. Thus we can perform a lifespan assay three times in 50 days using the *daf-16* mutant (twice in our previous method), suggesting that the number of agents tested could be increased approximately 1.5 fold in a lifespan assay using the *daf-16* mutant.

On the other hand, we unexpectedly found that reductions of lifespans by prolonged exposure to six ecotoxic agents were basically comparable between both strains (Table 2). In addition, enhanced toxicity in the *daf-16*(*mu86*) mutant was not detected in our short-term toxicity tests for reproduction and larval growth in comparison with those in the wild-type animals (Table 1 and Fig. 1). These results are consistent with previous observations from phenotypic analyses of the *daf-16*(*m*26) mutant with a different allele. For instance, Murakami and Johnson[12] observed that the *daf-16*(*m*26) mutant and wild-type nematodes exhibited similar sensitivities to UV irradiation, and Barstyte *et al.*[14]

Table 2. Lifespan Assay for Six Ecotoxicants

Toxicant tested	Mean lifespan ± S.E. (days)	
	N2 strain	CF1038 strain
$CuSO_4$ (mM) 0	25.7 ± 0.6 (n = 32)	18.7 ± 0.6 (n = 35)
0.1	24.5 ± 0.7 (n = 33)	16.4 ± 0.7 (n = 35)
0.3	18.4 ± 0.8 (n = 34)	14.9 ± 0.7 (n = 35)
1.0	15.5 ± 0.7 (n = 36)	12.3 ± 0.5 (n = 36)
$CdCl_2$ (mM) 0	25.5 ± 0.6 (n = 33)	19.2 ± 0.5 (n = 33)
0.03	20.3 ± 0.7 (n = 32)	16.1 ± 0.7 (n = 35)
0.1	18.5 ± 0.8 (n = 33)	15.1 ± 0.8 (n = 33)
0.3	10.7 ± 0.6 (n = 36)	6.8 ± 0.3 (n = 36)
SDS (%) 0	28.6 ± 0.6 (n = 32)	20.8 ± 0.4 (n = 34)
0.001	27.2 ± 0.6 (n = 33)	20.3 ± 0.5 (n = 29)
0.003	16.8 ± 1.2 (n = 33)	15.6 ± 0.7 (n = 31)
0.01	1.0 (n = 33)	1.0 (n = 33)
Detergent (%) 0	35.7 ± 1.0 (n = 32)	18.6 ± 0.5 (n = 28)
0.0033	16.5 ± 0.8 (n = 31)	14.6 ± 1.0 (n = 27)
0.01	4.6 ± 0.2 (n = 31)	4.7 ± 0.2 (n = 32)
0.033	3.1 ± 0.1 (n = 33)	2.8 ± 0.1 (n = 33)
0.1	2.0 ± 0.1 (n = 33)	1.7 ± 0.1 (n = 33)
PFOA (mM) 0 (0.5% DMSO; control)	27.1 ± 0.4 (n = 38)	19.6 ± 0.5 (n = 37)
0.03	25.0 ± 0.4 (n = 36)	17.6 ± 0.6 (n = 36)
0.1	25.0 ± 0.5 (n = 35)	17.8 ± 0.7 (n = 27)
0.3	23.9 ± 0.5 (n = 34)	18.4 ± 0.6 (n = 34)
1.0	23.0 ± 0.7 (n = 34)	17.1 ± 0.6 (n = 36)
3.0	1.4 ± 0.1 (n = 36)	3.7 ± 0.5 (n = 45)
Dichlorvos (μM) 0 (0.5% DMSO; control)	25.4 ± 0.4 (n = 38)	18.2 ± 0.5 (n = 34)
3.1	23.5 ± 0.9 (n = 37)	18.3 ± 0.6 (n = 36)
9.4	21.3 ± 0.9 (n = 25)	14.8 ± 0.8 (n = 23)
31	8.2 ± 0.5 (n = 38)	7.0 ± 0.4 (n = 36)
94	2.9 ± 0.4 (n = 38)	2.6 ± 0.2 (n = 37)
310	1.9 ± 0.3 (n = 37)	1.1 ± 0.1 (n = 36)

Mean lifespan of nematodes in each group as shown in Figs. 2 and 3. All animals in the presence of 0.01% SDS died within 1 day. n: no. animals tested.

also obtained a consistent result among the strains by assessing toxicity with cadmium and copper in the same mutant. Taken together with these observations, our results suggest that DAF-16 does not largely contribute to resistance to the ecotoxicants tested here. This raises the question why dysfunction of DAF-16 does not affect the survival of adult nematodes very much in the presence of these toxicants. While the exact mechanisms involved are unknown, these phenomena may be explained by functional compensation by other transcription factors involved in stress resistance, such as heat-shock transcription factor HSF-1[23] and stress response transcription factor SKN-1.[24] These proteins may compensate for the *daf-16* defects to maintain the viability in the *daf-16* animals treated with ecotoxicants, because they are known to regulate several DAF-16-independent stress response genes. To date, enhanced sensitivity to toxicants in the *daf-16* mutant have not been observed; however, Chu *et al.*[25] reported that a double mutant, *daf-16 unc-75*, exhibited 2-6-fold enhancement of sensitivity to heavy metals over the wild-type nematode, whereas a single *daf-16* mutant did not, even though the mechanism of this phenomenon was unknown.

In this study, we showed toxic effects with four kinds of representative ecotoxicants (heavy metals, detergents, a perfluoro organic compound, and an organophosphate insecticide) on reproductive capacity in young adult nematodes, the growth of hatched larvae, and shortening of the lifespan of adult nematodes, and obtained consistent results

with our previous report[5] from these toxicity assays in both wild-type and *daf-16* mutant animals exposed to heavy metals and detergents (Table 1, Figs. 1 and 2). In addition, we examined the toxic effects of PFOA and dichlorvos in these endpoints in both strains. To date, no reports have described the long-term effects on nematode lifespan by prolonged exposure to perfluoro organic compounds and insecticides. Perfluoro organic compounds are widely used in various housekeeping products, and it has been recently observed that these chemicals accumulate in the environment, wildlife, and humans.[26] Since PFOA is stable and accumulates in animals, it is important for risk assessment of organic perfluorochemicals to examine the effects resulting from low-concentration and long-term exposure to PFOA. To date, perfluoro organic compounds have been extensively studied in various aspects of toxicity.[27] Tominaga *et al.*[28] reported that exposure to perfluoro organic compounds including PFOA causes lethality and disrupted fecundity in *C. elegans*. In our experiments, PFOA effectively inhibited reproductive activity and larval growth in both N2 and *daf-16* strains at concentrations > 1.0 mM (Table 1 and Fig. 1). The EC_{50} value of PFOA calculated from reduction in the growth rate of N2 larvae is 0.9 mM, which is slightly lower than the EC_{50}s of PFOA (2.35–3.85 mM) from acute lethal toxicity tests reported by Tominaga *et al.*[28] We observed drastic reduction of lifespans by continuous exposure to PFOA in the concentration range 1.0–3.0 mM (Fig. 3A and B). The EC_{50} values of PFOA [approximately 2.1 (CF1038 strain) and 2.0 mM (N2 strain)] in the lifespan assay are similar to those in our growth retardation assay or acute toxicity tests by others.[28] 1*H*,1*H*,2*H*,2*H*-perfluorodecanol (8 : 2 TFOH) is a polyfluorinated compound that is converted into PFOA in animals. We examined the effects on lifespan with 8 : 2 TFOH, but only partial reductions of lifespans were observed (data not shown), suggesting that PFOA converted from 8 : 2 TFOH in animals likely causes physiologically toxic effects *in vivo*. We further examined the effects of the insecticide dichlorvos on nematodes. Dichlorvos is a representative organophosphate that inhibits acetylcholinesterase activity in higher animals including *C. elegans*, and the resultant continuous excitation of motor neurons causes lethality. We detected a concentration-dependent shortening of the lifespan of adult nematodes by exposure to dichlorvos (Fig. 3C and D). Previous studies showed that dichlorvos inhibits motor function of nematodes at a low concentration (0.7 μM as EC_{50})[29] and the LC_{50} value of dichlorvos in an acute toxicity test was reported to be 39 μM in *C. elegans*.[30] The EC_{50} value in our lifespan assay was approximately 20 (CF1038 strain) and 23 μM (N2 strain), which is consistent with being above the LC_{50} value of dichlorvos. However, unexpectedly we failed to detect significant reductions of brood size (Table 1) and growth retardation of hatched larvae (Fig. 1G and H) by dichlorvos at concentrations > 31 μM. So far, it is unknown why this phenomenon was observed.

In this study, close correlation in the effective concentrations of toxicants between acute toxicities and shortening of lifespan was found in the toxicity tests for cadmium, SDS, and PFOA, suggesting that the lifespan of nematodes can be used as an endpoint for assessment of ecotoxicity caused by these agents. In addition, we should note that a lifespan assay using a short-lived *daf-16* mutant is a useful tool for assessing potential toxicity of chemicals due to its short assay period. Finally, some limitations in the lifespan assay using the *daf-16* mutant should be considered; for example, we observed possible low sensitivity of the *daf-16* mutant at a low concentration of dichlorvos as described. In this lifespan assay, agents that interfere in insulin/IGF-1 signaling pathway are not applicable. While the *daf-16* mutant did not exhibit enhanced sensitivities to the toxicants tested, use of *C. elegans* mutants with defects in stress tolerance or RNAi-treated nematodes to DAF-16-independent stress resistance genes may possibly improve the sensitivity in a toxicity assay based on lifespan measurement of *C. elegans*.

Acknowledgements We thank Dr. Isao Katsura (National Institute of Genetics) for the nematode strain, Dr. Nobuaki Tominaga (Ariake National College of Technology) for useful comments on perfluoro organic compounds, and Hiroaki Harada, Rie Hayashi, and other lab members for helpful discussions and technical support. We would like to thank the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR), for providing the *daf-16* strain. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and grants from the research program of Toyohashi University of Technology, the Foundation for Earth Environment, and the Sumitomo

Foundation (to T.E.).

REFERENCES

1) Sohlenius, B. (1980) Abundance, biomass and contribution to energy flow by soil nematodes in terrestrial ecosystems. *J. Nematol.*, **11**, 186–194.
2) Sochova, I., Hofman, J. and Holoubek, I. (2006) Using nematodes in soil ecotoxicology. *Environ. Int.*, **32**, 374–383.
3) Freeman, M. N., Marse, T. J. and Williams, P. L. (1998) Establishment of quality assurance procedures for aquatic toxicity testing with the nematode *Caenohabditis elegans*. In *Environmental Toxicology and Risk Assessment* (Little, E. E., Delonay, A. J., Greenberg, B. M.), American Society for Testing and Materials, Philadelphia, pp. 45–59.
4) Freeman, M. N., Peredney, C. L. and Williams, P. L. (1999) A soil bioassay using the nematode *Ceanorhabditis elegans*. In *Environmental Toxicology and Risk Assessment* (Henshel, D. S., Black, M. C., Harrass, M. C.), American Society for Testing and Materials, Philadelphia, pp. 305–318.
5) Harada, H., Kurauchi, M., Hayashi, R. and Eki, T. (2007) Shortened lifespan of nematode *Caenorhabditis elegans* after prolonged exposure to heavy metals and detergents. *Ecotoxicol. Environ. Saf.*, **66**, 378–383.
6) Hsin, H. and Kenyon, C. (1999) Signals from the reproductive system regulate the lifespan of *C. elegans*. *Nature*, **399**, 362–366.
7) Lin, K., Dorman, J. B., Rodan, A. and Kenyon, C. (1997) *daf-16*: An HNF-3/forkhead family member that can function to double the life-span of *Caenorhabditis elegans*. *Science*, **278**, 1319–1322.
8) Baumeister, R., Schaffitzel, E. and Hertweck, M. (2006) Endocrine signaling in *Caenorhabditis elegans* controls stress response and longevity. *J. Endocrinol.*, **190**, 191–202.
9) Murphy, C. T., McCarroll, S. A., Bargmann, C. I., Fraser, A., Kamath, R. S., Ahringer, J., Li, H. and Kenyon, C. (2003) Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*. *Nature*, **424**, 277–283.
10) Lin, K., Hsin, H., Libina, N. and Kenyon, C. (2001) Regulation of the *Caenorhabditis elegans* longevity protein DAF-16 by insulin/IGF-1 and germline signaling. *Nat. Genet.*, **28**, 139–145.
11) Ohkumo, T., Masutani, C., Eki, T. and Hanaoka, F. (2006) Deficiency of the *Caenorhabditis elegans* DNA polymerase η homologue increases sensitivity to UV radiation during germ-line development. *Cell Struct. Funct.*, **31**, 29–37.
12) Murakami, S. and Johnson, T. E. (1996) A genetic pathway conferring life extension and resistance to UV stress in *Caenorhabditis elegans*. *Genetics*, **143**, 1207–1218.
13) Lithgow, G. J. and Walker, G. A. (2002) Stress resistance as a determinate of *C. elegans* lifespan. *Mech. Ageing Dev.*, **123**, 765–771.
14) Barsyte, D., Lovejoy, D. A. and Lithgow, G. J. (2001) Longevity and heavy metal resistance in *daf-2* and *age-1* long-lived mutants of *Caenorhabditis elegans*. *FASEB J.*, **15**, 627–634.
15) Larsen, P. L. (1993) Aging and resistance to oxidative damage in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U. S. A.*, **90**, 8905–8909.
16) Vanfleteren, J. R. (1993) Oxidative stress and ageing in *Caenorhabditis elegans*. *Biochem. J.*, **292**, 605–608.
17) Johnson, T. E., Henderson, S., Murakami, S., de Castro, E., de Castro, S. H., Cypser, J., Rikke, B., Tedesco, P. and Link, C. (2002) Longevity genes in the nematode *Caenorhabditis elegans* also mediate increased resistance to stress and prevent disease. *J. Inherit. Metab. Dis.*, **25**, 197–206.
18) Ishii, N., Fujii, M., Hartman, P. S., Tsuda, M., Yasuda, K., Senoo-Matsuda, N., Yanase, S., Ayusawa, D. and Suzuki, K. (1998) A mutation in succinate dehydrogenase cytochrome *b* causes oxidative stress and ageing in nematodes. *Nature*, **394**, 694–697.
19) Kayser, E. B., Morgan, P. G. and Sedensky, M. M. (1999) GAS-1: a mitochondrial protein controls sensitivity to volatile anesthetics in the nematode *Caenorhabditis elegans*. *Anesthesiology*, **90**, 545–554.
20) Ogg, S. and Ruvkun, G. (1998) The *C. elegans* PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway. *Mol. Cell*, **2**, 887–893.
21) Ookuma, S., Fukuda, M. and Nishida, E. (2003) Identification of a DAF-16 transcriptional target gene, *scl-1*, that regulates longevity and stress resistance in *Caenorhabditis elegans*. *Curr. Biol.*, **13**, 427–431.
22) Nakamura, M., Ando, R., Nakazawa, T., Yudazono, T., Tsutsumi, N., Hatanaka, N., Ohgake, T., Hanaoka, F. and Eki, T. (2007) Dicer-related *drh-3* gene functions in germ-line development by maintenance of chromosomal integrity in *Caenorhabditis elegans*. *Genes Cells*, **12**, 997–1010.
23) Hsu, A. L., Murphy, C. T. and Kenyon, C. (2003) Regulation of aging and age-related disease by DAF-16 and heat-shock factor. *Science*, **300**, 1142–

1145.
24) An, J. H. and Blackwell, T. K. (2003) SKN-1 links *C. elegans* mesendodermal specification to a conserved oxidative stress response. *Genes Dev.*, **17**, 1882–1893.
25) Chu, K. W., Chan, S. K. and Chow, K. L. (2005) Improvement of heavy metal stress and toxicity assays by coupling a transgenic reporter in a mutant nematode strain. *Aquat. Toxicol.*, **74**, 320–332.
26) Hansen, K. J., Johnson, H. O., Eldridge, J. S., Butenhoff, J. L. and Dick, L. A. (2002) Quantitative characterization of trace levels of PFOS and PFOA in the Tennessee River. *Environ. Sci. Technol.*, **36**, 1681–1685.
27) Lau, C., Anitole, K., Hodes, C., Lai, D., Pfahles-Hutchens, A. and Seed, J. (2007) Perfluoroalkyl acids: a review of monitoring and toxicological findings. *Toxicol. Sci.*, **99**, 366–394.
28) Tominaga, N., Kohra, S., Iguchi, T. and Arizono, K. (2004) Effects of perfluoro organic compound toxicity on nematode *Caenorhabditis elegans* fecundity. *J. Health Sci.*, **50**, 545–550.
29) Cole, R. D., Anderson, G. L. and Williams, P. L. (2004) The nematode *Caenorhabditis elegans* as a model of organophosphate-induced mammalian neurotoxicity. *Toxicol. Appl. Pharmacol.*, **194**, 248–256.
30) Rajini, P. S., Melstrom, P. and Williams, P. L. (2008) A comparative study on the relationship between various toxicological endpoints in *Caenorhabditis elegans* exposed to organophosphorus insecticides. *J. Toxicol. Environ. Health A*, **71**, 1043–1050.