

# A Multi-Generation Sublethal Assay of Phenols Using the Nematode *Caenorhabditis elegans*

Nobuaki Tominaga,<sup>\*,a</sup> Shinya Kohra,<sup>b</sup> Taisen Iguchi,<sup>c,d</sup> and Koji Arizono<sup>c,e</sup>

<sup>a</sup>Department of Chemical and Biochemical Engineering, Ariake National College of Technology, 150 Higashi-hagio-machi, Omuta, Fukuoka 836–8585, Japan, <sup>b</sup>Faculty of Environmental Sciences, Nagasaki University, 1–14 Bunkyo-machi, Nagasaki 852–8521, Japan, <sup>c</sup>CREST, JST, Japan Science and Technology Corporation, 4–1–8 Honmachi, Kawaguchi, Saitama 332–0012, Japan, <sup>d</sup>Department of Bio-environmental Research, Center for Interactive Bioscience, National Institute for Basic Biology, 5–1 Higashiyama Myodaiji, Okazaki, Aichi 444–8585, Japan, and <sup>e</sup>Faculty of Environmental and Symbiotic Sciences, Kumamoto Prefectural University, 3–1–100 Tsukide, Kumamoto 862–8502, Japan

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The nematode *Caenorhabditis elegans* (*C. elegans*) was used for a multiple-generation toxicity bioassay of phenols. We examined the sublethal toxicity (fecundity and reproduction) of bisphenol A (BisA), 4-(1-adamantyl) phenol (Adp), and 4,4'-(1,3-adamantanediyl) diphenol (AdDP) over five generations using a Nematode Growth Medium (NGM) 1.7% agar plate. In the fourth generation, the phenols affected the fecundity rate of *C. elegans* at doses 100- to 10000-fold lower than the LC<sub>50</sub>s. In particular, at 1 nM BisA, the number of worms decreased significantly to about 50% of control ( $p < 0.05$ ). A comparison of the number of viable worms and eggs suggested that the phenols exert hatching toxicity. In addition, individuals with an abnormality in the vulva, which could not ovulate, appeared on the phenol-containing plates. We confirmed that phenols disrupt reproduction in *C. elegans* and that the method using NGM agar plates facilitates multi-generational toxicity tests of chemicals that are poorly soluble in water.

**Key words** — *Caenorhabditis elegans*, reproduction, fecundity, reproductive disruption

## INTRODUCTION

Recently, endocrine disrupting chemicals (EDCs) have received increasing attention from ecotoxicologists.<sup>1)</sup> An unimaginable number of chemicals are released daily into the environment, and many of these chemicals are suspected to have endocrine-disrupting activity and thereby affect organisms in many ecosystems. Hence, there has been an increasing need for a rapid assay system to evaluate the toxicity of chemicals at lower concentrations.

*Caenorhabditis elegans* (*C. elegans*) is a free-living nematode with a thin, transparent body approximately 1 mm in length, which lives mainly in the liquid phase of soils. The genome of this organism is completely mapped,<sup>2)</sup> and it has become a commonly used laboratory model for molecular and cellular biology owing to its short life cycle (3 days

at 20°C) and ease of rearing in the laboratory.<sup>3)</sup> Many studies have used *C. elegans* in assessments of acute and chronic chemical hazards,<sup>4–14)</sup> and thus *C. elegans* serves as an efficient model for testing the effects of xenobiotic chemicals *in vivo*. Accordingly, we have established several laboratory-scale bioassay systems to evaluate the impact of various chemicals on *C. elegans*.<sup>15–18)</sup>

Nonylphenol (NP) and bisphenol A (BisA) are known to bind estrogen receptors. Yamakoshi *et al.* reported previously that 4-(1-adamantyl) phenol (AdP) and 4,4'-(1,3-adamantanediyl) diphenol (AdDP) exhibited potent estrogenic activity.<sup>19)</sup> In the present paper, we investigated the sublethal effects of BisA, AdP, and AdDP over five generations using fecundity, reproduction, and physical abnormality as toxicity endpoints.

## MATERIALS AND METHODS

**Chemicals** — Agar, dimethyl sulfoxide (DMSO), Tryptone, sodium chloride, and BisA were purchased from Wako Pure Chemicals (Japan). Reagent grade

\*To whom correspondence should be addressed: Department of Chemical and Biological Engineering, Ariake National College of Technology, 150 Higashihagio-machi, Omuta, Fukuoka 836–8585, Japan. Tel.: +81-944-53-8747; Fax: +81-944-53-8758; E-mail: tominaga@ariake-nct.ac.jp

AdP and AdDP were supplied by Aldrich.

**Animals and Culture Conditions** — The wild-type N2 strain of *C. elegans* was used in the present study. Worms were maintained and handled as described by Brenner.<sup>20</sup> Briefly, 3 to 5 worms were grown on a nematode growth medium (NGM) 1.7% agar plate with a lawn of *Escherichia coli* (*E. coli*) DH5 $\alpha$  as a food source and incubated at 20°C. Every 4 or 5 days, worms were sub-cultured to new plates.

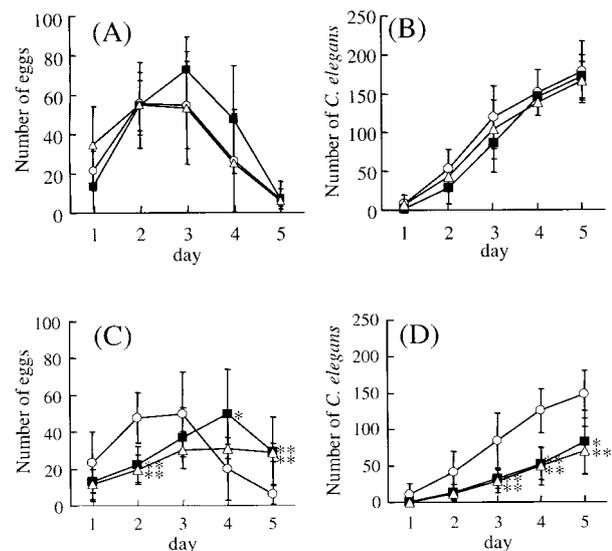
**Multi-Generation Test** — DMSO was used as the toxicant vehicle at a final concentration of 0.5%.<sup>17</sup> The test chemicals in DMSO were added to autoclaved NGM 1.7% agar medium. After the mixture was sonicated, the warm medium was poured into the petri plates and allowed to harden. Subsequently, *E. coli* DH5 $\alpha$  was spread on the plate. Approximately 20 *C. elegans* eggs, obtained by alkali-bleaching method,<sup>5</sup> were placed on each plate and incubated at 16°C. One L4 larva was then transferred to a separate plate and defined as the first generation. Four replicates were set up for each treatment. The worms and eggs on each plate were counted under a dissecting microscope at a fixed time every day. The day that the first offspring was identified was defined as the first day. When the second generation worms attained the L4 stage, one worm was sub-cultured to a new plate. The above steps were repeated until the fifth generation was cultured. These experiments were carried out at least twice.

**Statistical Analyses** — Where applicable, data are reported as the mean  $\pm$  S.E.M. Statistical significance was evaluated by analysis of variance (ANOVA). Values of  $p < 0.05$  were considered statistically significant.

## RESULTS AND DISCUSSION

Previously, we demonstrated that an acute toxicity assay using *C. elegans* as a model organism is a convenient and easy method for evaluation of chemical substances.<sup>17,18</sup> Others have also proposed the use of *C. elegans* for evaluating aquatic or soil toxicity. However, water insoluble or slightly soluble chemicals could not be evaluated in many cases because of the experimental conditions.

Previously, we reported that *C. elegans* responded to specific steroids in reproduction and fecundity rates.<sup>21</sup> In that experiment, *C. elegans* were exposed to various steroids on an agar plate. In the present paper, we investigated the applicability of



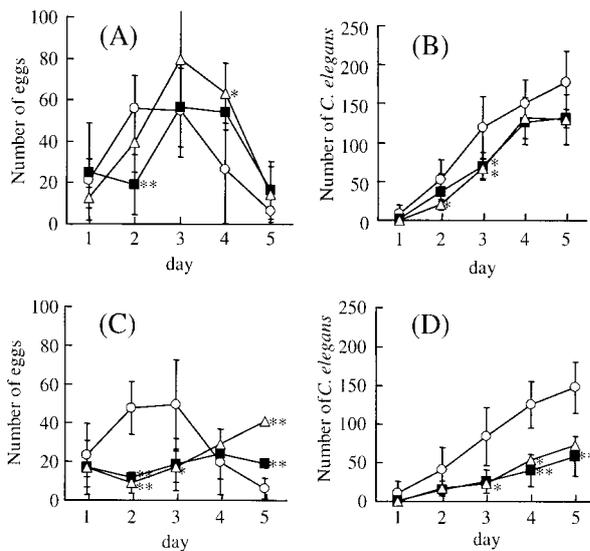
**Fig. 1.** Effects of Bis A on the Fecundity of *C. elegans*

(A) Egg abundance at 1st generation, (B) *C. elegans* abundance at 1st generation, (C) egg abundance at 4th generation, (D) *C. elegans* abundance at 4th generation. ○, Control; ■, 1 nM; △, 10 nM.

our test system to low solubility or suspected endocrine disrupting chemicals. We tested three phenols, BisA, AdP, and AdDP. Figure 1 summarizes the results of the assay using *C. elegans* exposed to BisA on a NGM 1.7% agar plate containing 0.5  $\mu$ M cholesterol. First generation and control worms produced a similar number of eggs [Fig. 1(A)]. In contrast, exposure to BisA delayed the peak egg abundance [Fig. 1(C)]. The fertilized egg of *C. elegans* is an ellipsoid shape because of cell polarity, whereas an unfertilized egg is globular in shape. The shape of ovulated eggs in these exposures suggested that few were fertilized.

Worm abundance during the first generation did not differ significantly from controls [Fig. 1(B)]. However, by days 3–5 of the fourth generation, abundance had decreased to about 50% of controls [Fig. 1(D)]. The abundance of viable worms and eggs in these exposures suggested that BisA exerts toxic effects on hatching. Similar effects were observed in response to nonylphenol.<sup>16</sup> The BisA 50% lethal concentration (LC<sub>50</sub>) value for *C. elegans* was over 100  $\mu$ M.<sup>18</sup> In our experiments, sublethal effects of BisA on *C. elegans* were observed at 1 and 10 nM, levels that are over 10000-fold lower than the LC<sub>50</sub> value. On 100 nM BisA plates, *C. elegans* lost fecundity, and we could not culture them after the third generation.

The effects of AdP on *C. elegans* fecundity are shown in Fig. 2. AdP exposure affected egg abun-

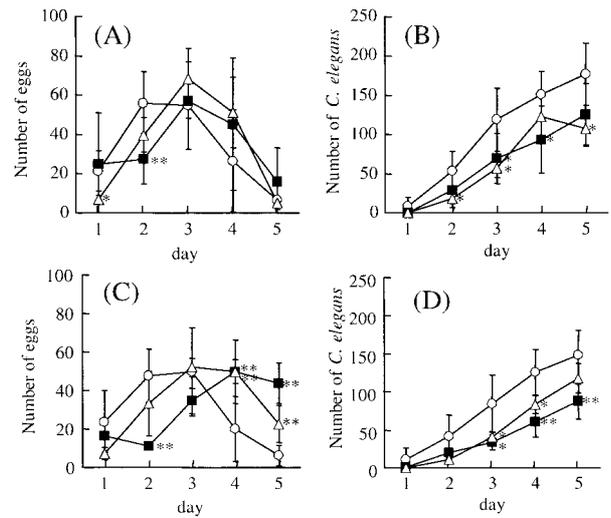


**Fig. 2.** Effects of AdP on the Fecundity of *C. elegans*

(A) Egg abundance at 1st generation, (B) *C. elegans* abundance at 1st generation, (C) egg abundance at 4th generation, (D) *C. elegans* abundance at 4th generation. ○, Control; ■, 10 nM; △, 100 nM.

dance patterns, and the effects on the fourth generation were more severe than on the first generation [Figs. 2(A) and 2(C)]. The abundance of worms in the first generation decreased by about 15% from the control levels at 10 nM or 100 nM AdP [Fig. 2(B)], and AdP exerted its effects faster than BisA. Moreover, at 10 nM AdP, the fourth generation of viable worms decreased to about 40% of control ( $p < 0.01$ ) [Fig. 2(D)]. Our research showed that exposure to greater than 10 nM of AdDP decreased fecundity to the same extent as AdP (Figs. 2 and 3). However, the effects of high concentration and low concentration exposures to AdDP did not differ significantly and a dose-response relationship was not observed. Chu *et al.*<sup>14)</sup> reported that dose-response relationship was not observed in some case of lethal toxicity of metals on *C. elegans*. The dose response curves of copper and zinc in mortality rate on *C. elegans* did not show the dose dependent manner at low concentration. We were unable to demonstrate reproductive toxicity below 1 nM BisA, AdP, or AdDP using this NGM 1.7% agar plate bioassay system.

We previously reported that worms occasionally exhibited an abnormal vulva when exposed nonylphenol.<sup>16)</sup> In the present study, in all exposed groups also occasionally individuals exhibited an abnormality in the vulva (Fig. 4). Greenwald *et al.* reported that *lin-12(null)* mutant, *lin-12(n137 n720)*, had a large protrusion at the normal position of the



**Fig. 3.** Effects of AdDP on the Fecundity of *C. elegans*

(A) Egg abundance at 1st generation, (B) *C. elegans* abundance at 1st generation, (C) egg abundance at 4th generation, (D) *C. elegans* abundance at 4th generation. ○, Control; ■, 10 nM; △, 100 nM.



**Fig. 4.** Photographs of Normal Hermaphrodite (A) and Individual with Large Protrusion at the Normal Position of the Vulva (B), which was Exposed to AdP

vulva<sup>22)</sup> similar to the result of our experiment. The *lin-12* is a gene that specifies the fates of cells with fates controlled by cell interaction. These facts and our results suggest that phenolic chemicals may ex-

ert disruptive effects on fates of cell and effect on reproduction. We have been used cDNA microarrays to explore the variation in expression of approximately 5000 unique genes of *C. elegans* exposed chemicals including BisA. When the analysis is finished, more information is obtained and the point of application may be clarified.

Although BisA is nearly insoluble in water (BisA 120–300 mg/l), the NGM 1.7% agar plates appear to provide an effective vehicle for multi-generational toxicity tests using water-insoluble chemicals.

In conclusion, we examined the effects of BisA, AdP and AdDP on *C. elegans* over five generations and clarified their sublethal toxicity at a concentration of about 1/100 to 1/10000 of LC<sub>50</sub> using fecundity, reproduction, growth and physical abnormality as toxicity endpoints. And the chemicals appear to have some hatching toxicity.

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