A Convenient Sublethal Assay of Alkylphenol and Organotin Compounds Using the Nematode *Caenorhabditis elegans*

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The free-living nematode, *Caenorhabditis elegans* (*C. elegans*) was adopted as a multicellular biosensor of biological toxicity from alkylphenols and organotin compounds. Alkylphenols were found to affect reproduction at lower doses than indicated by the acute toxicity assay. In particular, nonylphenol altered the reproduction rate of *C. elegans* at a dose 10- to 100-fold lower than the 50% lethal concentration (LC_{50}). A comparison of the number of viable worms and eggs suggested that alkylphenols and organotin compounds possess hatching toxicity. A 0.1 μM dose of organotin compounds caused a significant decrease, in the order of 20–50%, in reproduction of the worms, and an abnormal male: hermaphrodite ratio was observed. *C. elegans* therefore appears to represent a potent and sensitive organism with which to evaluate the biological effects of chemicals. In particular, the sensitivity of reproduction as an endpoint is highly useful for assessing the sublethal effects of chemicals.

Key words —— *Caenorhabditis elegans*, reproduction, fecundity, hatching rate, male expression, biosensor

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INTRODUCTION

Various organisms and toxicological endpoints have been evaluated for their suitability to assess the toxicity of chemical substances. Several criteria must be fulfilled for an indicator organism to be adopted as a biosensor. The organism must be sensitive to the testing toxicants, easy to manage in the laboratory and available throughout the year. The free-living nematode, *Caenorhabditis elegans* (*C. elegans*), widely used as a simple multicellular organism in developmental biology studies, satisfies all these criteria,[1–3] and its culture conditions, developmental staging, anatomy and genetic properties are well defined. In addition, researchers can take advantage of the worm's short life cycle, low cost and little individual variation. Moreover, genomic sequencing of *C. elegans* has recently been completed.[4] Accordingly, we believe that *C. elegans* will become a more potent model organism for basic and applied research. However, at present, only few research groups use *C. elegans* as a model organism for bioassays.[5–9]

In a previous study, we observed and evaluated the feeding behavior of *C. elegans* after exposure to bisphenol A and nonylphenol[10] and constructed a convenient and acute toxicity assay (Tominaga, N., Kai, T., Kunimoto, M., Arizono, K. and Kohra, S., unpublished results). We also demonstrated that fecundity and reproduction rates are good endpoints as biomarkers and also evaluated the generational effects of steroids and synthetic hormones on *C. elegans* (Tominaga, N., unpublished results).

In the present study, we investigated the sublethal effects of alkylphenols and organotin compounds using the reproduction and fecundity of *C. elegans* as indicators.

MATERIALS AND METHODS

Chemicals —— Cholesterol (Cholesterin) was purchased from Nacalai Tesque, Inc. Kyoto, Japan. 4-Nonylphenol (NP), *p*-octylphenol (OP), triphenyltin chloride (TPT) and tributyltin chloride (TBT) were purchased from Kanto Chemical Co., Inc. Tokyo, Japan. All chemicals used were analysis grade.

Preparation of Nematode Cultures —— The wild-type nematode *C. elegans* was used and was maintained on nematode growth medium (NGM) plates, that had been was seeded with *Escherichia coli* (*E. coli*), at 20°C as described.[1]

Reproduction and Fecundity Test —— The forth instar larvae (L4) were transferred using wormpick to NGM agar plates containing various concentrations of the test chemicals, 50 μg/ml cholesterol and a lawn of *E. coli* in 6 cm dishes. These experimental plates consisted of 3 concentrations (0.02 μM, 0.2 μM, or 2.0 μM) of NP, 3 concentrations (0.02 μM, 0.2 μM, or 2.0 μM) of OP, 2 concentrations (0.01 μM, 0.1 μM) of TPT and 2 concentrations (0.01 μM or 0.1 μM) of TBT. *E. coli* was grown circularly within a 1 cm radius from the center. The plates were then incubated at 16°C. Worms and eggs on the plates were counted under a dissecting microscope at a fixed time daily. When the next generation of worms had grown to L4 larvae, one worm was picked up using wormpick and sub-cultured on a new plate containing the same ingredients. At 16°C, each step in the lifecycle of *C. elegans* takes about one day and the organism grows into adult from an egg in 5–6 days. This culturing was continued until the third-generation. Five replicates for each treatment were set-up and the test was repeated at least twice.

Statistics —— Results are presented as means ± S.E. of at least 10 warms in each group. Student's *t*-test was used to calculate the significance of difference. All statistical calculations were performed with StatView-J 5.0 software (SAS Institute, Inc., Cary, NC, U.S.A.), with $p < 0.05$ being required for significance.

RESULTS AND DISCUSSION

Previously, we described a convenient acute toxicity assay using *C. elegans* larvae and evaluated the acute toxicity of alkylphenol compounds. *C. elegans* was relatively sensitive to the alkylphenol compounds in that acute toxicity test, with 50% lethal concentration (LC_{50}) values of 5 μM for NP and 1.7 μM for OP obtained. In general, the lethal toxicities of the organotin compounds for *C. elegans* are stronger than those of alkylphenols. Consequently, in the present study, the exposure concentrations chosen were NP; 0.02 μM, 0.2 μM and 2.0 μM, OP; 0.02 μM, 0.2 μM and 2.0 μM, TBT; 0.01 μM and 0.1 μM, and TPT; 0.01 μM and 0.1 μM.

The reproduction rate was expressed as the number of worms on a plate and fecundity rate was expressed as the number of eggs on a plate. The findings for each 5-day course for each chemical for each generation are shown in Fig. 1. Figure 2 shows the fecundity findings for each 5-day course for each chemical for each generation. The value for 2.0 μM OP represents that of the first generation only, as after the worms were transferred from the first generation plate to the next new plates, all worms died on all replicate 2.0 μM OP plates within 24 hr. This result is not entirely unexpected and supports our previous finding that the LC_{50} of OP was 1.7 μM in liquid culture (Tominaga, N., Kai, T., Kunimoto, M., Arizono, K. and Kohra, S., unpublished results). This is evidently also true in plate culture, and indicates that OP is very strongly toxic for *C. elegans*.

It seemed that the effect of alkylphenols on reproduction was more obvious in the 2nd and 3rd generations than the 1st one (Fig. 1). The numbers of worms decreased in the 2nd and 3rd generations when exposed to the alkylphenols, and in particular 2.0 μM NP decreased the numbers of worms to 40–55% of the control number. For OP, by the 4th day of the 3rd generation the number of worms had decreased by 40%. The organotin compounds TPT and TBT both produced no significant difference in reproduction at 0.01 μM compared to the control. On the other hand, at 0.1 μM TPT and TBT, the worms showed a significant decrease in reproduction to 40–50% of control worm numbers. The reproduction rates in *C. elegans* were altered by a dose 2- to 200-fold lower than the LC_{50} (5 μM).

Compared Fig. 1 with Fig. 2, at NP, it seemed that the decrease of the number of viable worms had been caused by the decrease in the number of eggs. However, in the first generation, at 2.0 μM NP, the viable worms decreased to about 60% of control with no significant difference in total number of eggs. The similar phenomena were observed at 0.1 μM TBT of 2nd generation, 0.2 μM OP and 0.1 μM TPT of 3rd generation. Judging from the results, these decrease in reproduction result from not only the decrease of fecundity rate but also decrease of hatching rate. At 0.01 μM TBT of 3rd generation, total number of eggs (202 ± 14) at the 4th day was 30% higher than the control (152 ± 12), but the numbers of viable worms of expose group and control at the 5th day were not shown the significant difference. This phenomenon was even pronounced upon exposure to 2.0 μM OP, with the number worms completely ceasing to increase after the 2nd day, but with an accompanying increase in the number of eggs. The hatching rate was obtained by dividing the number of viable worms by the total number of eggs on the plate. Considered the hatching rates which were obtained by dividing the 5th day number of viable worms by the 4th day total number of eggs, the hatch-

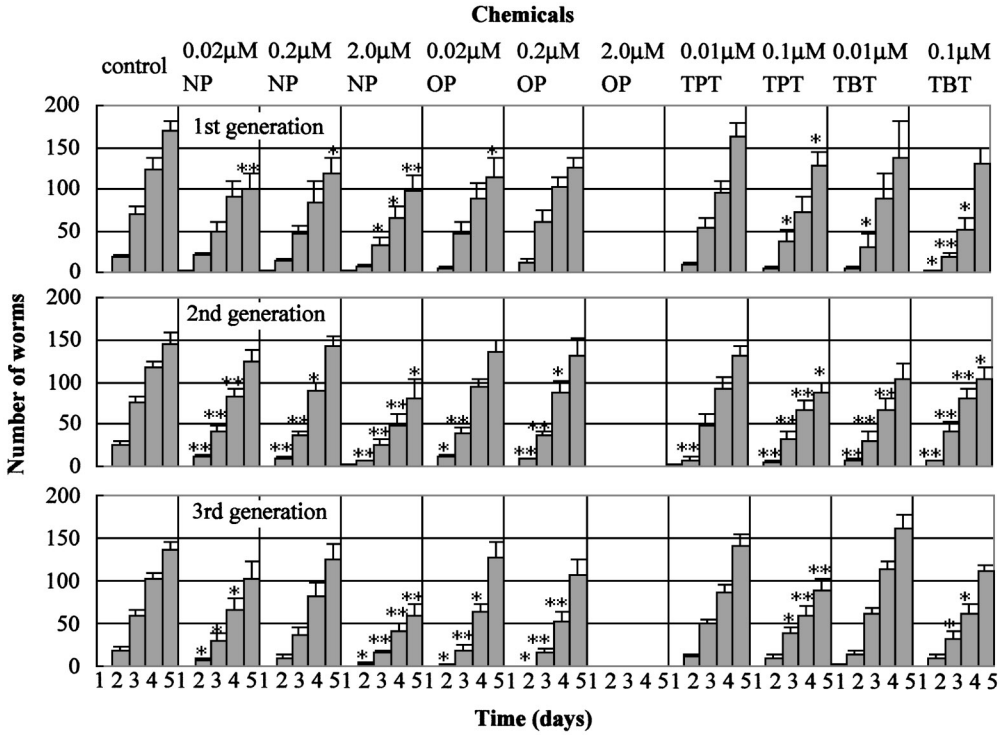

Fig. 1. Effect of Alkylphenols and Organotin Compounds on Reproduction of *C. elegans*

*$p < 0.05$, **$p < 0.01$. Error bar represents SEM ($n = 10$). Worms were incubated at 16°C. Worms on the plates were counted under a microscope at a fixed time daily. When the next generation worms had grown to L4 larvae, one worm was transferred and subcultured on a new plate containing the same ingredients.

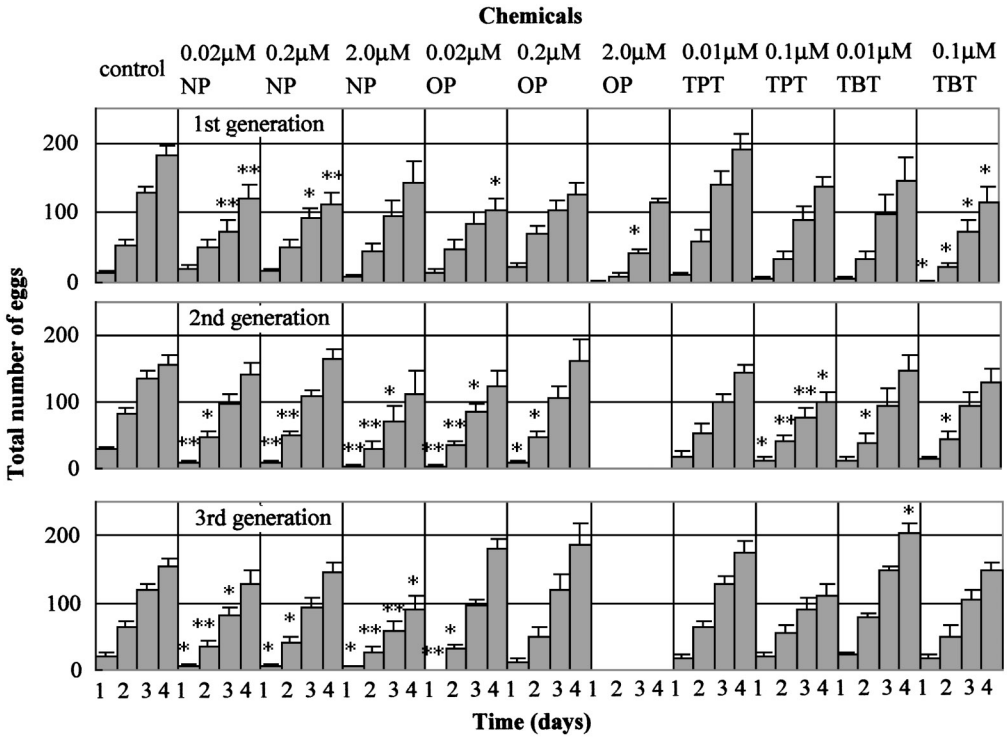

Fig. 2. Effect of Alkylphenols and Organotin Compounds on Fecundity of *C. elegans*

*$p < 0.05$, **$p < 0.01$. Error bar represents SEM ($n = 10$). Worms were incubated at 16°C. Eggs on the plates were counted under microscopy at a fixed time daily. When the next generation worms had grown to L4 larvae, one worm was transferred and subcultured on a new plate containing the same ingredients.

Fig. 3. Male Nematode

ing rates of the organism treated with chemicals were over 20% lower than that of control significantly ($p < 0.05$) at 2.0 μM NP on 1st generation, 2.0 μM NP and 0.01 μM TBT on 2nd generation, and 0.2 μM OP on 3rd generation. Since the fertilized egg of *C. elegans* has cell polarity, it takes an ellipsoid shape, on the other hand, the unfertilized egg takes a globular shape. Judging from the shape of ovulated egg, the unfertilized egg was very few. Thus, new worms did not hatch from the eggs, suggesting that alkylphenols and organotin compounds possess some toxicity for hatching.

Exposure to organotin compounds frequently led to shortened experiments as the subcultured worms were often male, resulting in insufficient data for analysis. Compared with the usual male manifestation frequency (1 male per 1000 worms), this phenomenon is stochastically rare, and thus an increase in male expression was indicated. This was confirmed in the remarkable example of 30 males born in 196 larvae from 1 hermaphrodite on 0.1 μM TBT plate (Fig. 3). In addition, on alkylphenols plates, individuals with an abnormality in the vulva were confirmed in the 3rd generation (Fig. 4), the incidence of the abnormality was about 0.5–1%. These findings indicate the need for further experimentation.

In conclusion, we examined the toxicity of alkylphenols and organotin compounds over three generations and clarified their sublethal effects at a concentration of about 1/100 of the LC_{50}. In addition, the chemicals appear to have some hatching toxicity. Interestingly, a morphological abnormality was observed under microscopic observation in the 3rd generation. The possibility arises of using the ratio of males to hermaphrodite and the accompanying morphological abnormality as endpoints in this bioassay in addition to reproduction and fecundity. Taken together, these results demonstrate the many merits of *C. elegans* as a potent tool for evaluation of chemical effects on living organisms.

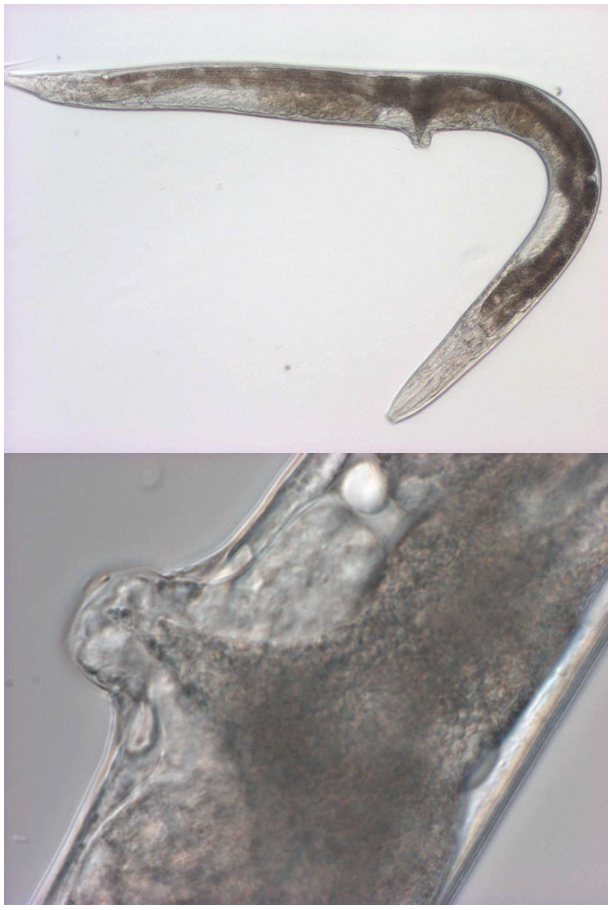

Fig. 4. Individual with an Abnormality in the Vulva

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