FULL PAPER *Toxicology*

Effects of 17β-Estradiol, Bisphenol A and Tributyltin Chloride on Germ Cells of *Caenorhabditis elegans*

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ABSTRACT. Effects of a one-generation exposure to a natural estrogen, 17β-estradiol (E2), and environmental pollutants such as bisphenol A (BPA) and tributyltin chloride (TBTCL) on the number of germ cells were investigated in the hermaphrodite *Caenorhabditis elegans*. The eggs of gravid adult worms isolated by alkaline hypochlorite treatment were seeded on a test chemical-containing NGM (nematode growth medium) agar plate without cholesterol. After incubation for 6 days at 16°C, the germ cells of adult worms were stained with 4', 6-diamino-2-phenylindole dihydrochloride (DAPI). The staining procedure was completed within one hour and the stained germ cells were counted under a fluorescence microscope without dissection. The number of germ cells in the worms treated with E2 (10^{-10}–10^{-6} M) and BPA (10^{-9}–10^{-5} M) was significantly increased. Maximal increases were observed at 10^{-8} M E2 (156 ± 15.3% of control) and 10^{-5} M BPA (168 ± 20.0 % of control). TBTCL (10^{-9}–10^{-6} M) significantly decreased the number of germ cells. The minimal decrease was observed at 10^{-6} M TBTCL (30.2 ± 3.51% of control). These results indicate that changes in the number of germ cells are a sensitive indicator of the effects of chemicals on the reproductive system. Since the method described in this paper is a novel, simple, time- and money-saving bioassay, *C. elegans* is an excellent model with which to determine the reproductive toxicity of chemicals including environmental pollutants.

KEY WORDS: bioassay, *Caenorhabditis elegans*, environmental pollutant, germ cell, reproductive toxicity.

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The possible adverse effects of synthetic chemicals on reproductive organs are currently cause for great concern. Among such chemicals, bisphenol A (BPA) and tributyltin (TBT) are commonly detected in the environment [16, 26, 33, 35] and in food products [5, 36]. BPA is the monomer component of polycarbonate plastics and epoxy resins. TBT, the active ingredient in antifouling paint, is a causative agent of imposex in female sea snails [3].

It was reported that BPA caused the proliferation of MCF-7 cells (human breast cancer cell line) [10] and induced the expression of vitellogenin (yolk protein precursor) in cultured fish hepatocytes [7]. TBT induces the superimposition of male organs such as the penis and/or vas deferens onto the female genital system, which is known as imposex [13, 32].

To determine the effects of chemicals on reproductive organs, uterotrophic assays in rats [1], MCF-7 cell proliferation assays [19], estrogen receptor binding assays [23] and two-hybrid assays [12] have been used. While *in vitro* assays can be performed quickly and reproducibly, the findings are less relevant to whole organisms. On the other hand, *in vivo* assays provide information relevant to animals, but are more cost- and time-intensive.

The free living nematode *Caenorhabditis elegans* is a small, rapidly growing organism that is easily maintained in the laboratory, where it can be grown on agar plates or in liquid culture with *Esherichia coli* as a food source. *C. elegans* may serve as a new model for determining the effects of chemicals on whole organisms because it is convenient and inexpensive to maintain. Additionally, *C. elegans* is well studied genetically, and much is known about its cellular anatomy, cell lineage, and neuronal wiring. This background is advantageous for investigations into the toxic mechanisms of chemicals. Recently, some attempts to detect estrogenic activities using vitellogenin mRNA in *C. elegans* were reported [9, 17]. However, morphological changes to *C. elegans* after exposure to E2 were not elucidated.

In this paper, we report a method of determining the one-generation effects of E2, BPA and TBTCL on the reproductive organs of *C. elegans*.

MATERIALS AND METHODS

Chemicals: Bacto-peptone and Bacto-agar were purchased from Becton Dickinson and Company (Sparks, MD). The 1, 3, 5 [10]-estratriene-3, 17β-diol (E2) was purchased from Sigma Chemical Co. (St. Louis, MO), while 2, 2-bis (4-hydroxyphenyl) propane (BPA) and tributyltin (IV) chloride (TBTCL) were obtained from Wako Pure Chemical Industry, Ltd (Osaka, Japan). 4', 6-diamino-2-phenylindole dihydrochloride (DAPI) was purchased from Nakalai Tesque, Inc. (Kyoto, Japan).

Nematode and E. coli strains: Bristol strain N2 *C. elegans* and *Escherichia coli* OP50 were kindly provided by Dr. Sawa (Osaka University, Japan).

Worms were maintained on NGM (nematode growth medium) agar plates (51.3 mM NaCl, 0.25 % Bacto-peptone, 1.7% Bacto-agar, 0.0005% cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$ and 25 mM potassium phosphate, pH 6.0) seeded with *E. coli* OP50 as described by Brenner [4]. Experiments were done at 16°C.

Chemical exposure: Age-synchronous worms were obtained from eggs following the treatment of gravid adults with alkaline hypochlorite as described [11]. The eggs were seeded on the testing agar plates containing E2, BPA, TBTCL or solvent only. The compounds were dissolved in ethanol (final concentration, 0.3% v/v) and were added to NGM agar without cholesterol before polymerization. The final concentrations of chemicals in the agar were 10^{-11}–10^{-6} M (E2), 10^{-10}–10^{-5} M (BPA) and 10^{-10}–10^{-6} M (TBTCL). The control worms were incubated on solvent only plates. The solvent control plates and agar test plates were also seeded with *E. coli* OP50.

Counting the germ cells by DAPI staining: After exposure to chemicals for 6 days, the worms were harvested from the surface of the agar plates in M9 buffer (42.3 mM Na_2HPO_4, 22 mM KH_2PO_4, 85.6 mM NaCl and 1 mM $MgSO_4$). Adult worms were separated from L1-L4 larvae and eggs with a 50-μm nylon mesh, and were washed with M9 buffer by centrifugation at 1,000-× g twice for 2 min. The collected worms were incubated in 500 *n*g/m*l* of DAPI (in ethanol) for 10 min, and were centrifuged at 1,000-× g for 2 min. The worms were suspended in distilled water, centrifuged at 1,000-× g for 2 min, and mounted on a slide glass with an agarose pad. The germ cells of adult hermaphrodites were observed under a fluorescence microscope (Nikon OPTIPHOT XF-EF, Nikon Co., Tokyo, Japan) and photographed (Nikon H-III, Nikon Co.). The stained germ cells were counted manually [2, 6].

Data analyses: A relative percentage of the number of germ cells (RPG) was calculated using the equation: RPG= (number of germ cells per worm)/(mean number of germ cells per 5 control worms) × 100. Each value given in Fig. 3 is expressed as the mean ± S.D. of 5 worms. Data were analyzed using Dunnett's multiple comparison test to compare each treatment group with the control. A value of $p<0.05$ was considered significant in the analyses.

RESULTS

Environmental pollutants, including xenoestrogens, were assessed for their toxic effects on the reproductive organs of *C. elegans*, which were evaluated by counting the germ cells after chemical exposure to determine whether *C. elegans* was a suitable experimental model.

The anatomy of the hermaphrodite gonad has been described in detail [37]. The adult hermaphrodite gonad consists of two U-shaped tubular arms as illustrated in Fig. 1. Nucleic DNA of *Caenorhabditis elegans* was stained with DNA-binding dye, DAPI. The DAPI-stained nucleus of germ cell in the gonad is large and bright as indicated in Fig. 1. According to our procedure, the one-generation effects of the chemicals on the reproductive organs of *C. elegans* could be estimated within 6 days. DAPI-staining of the germ cells was completed within one hour. Although the worm was mounted on a slide glass without dissection, the staining was clear as shown in Fig. 1. DAPI-stained images of the control worm and 10^{-8} M E2, 10^{-5} M BPA or 10^{-6} M TBTCL-treated worms were clearly different (Fig. 2). The numbers of germ cells in 10^{-8} M E2 and 10^{-5} M BPA-exposed worms were increased markedly, while the number in 10^{-6} M TBTCL-exposed worms was decreased (Fig. 2).

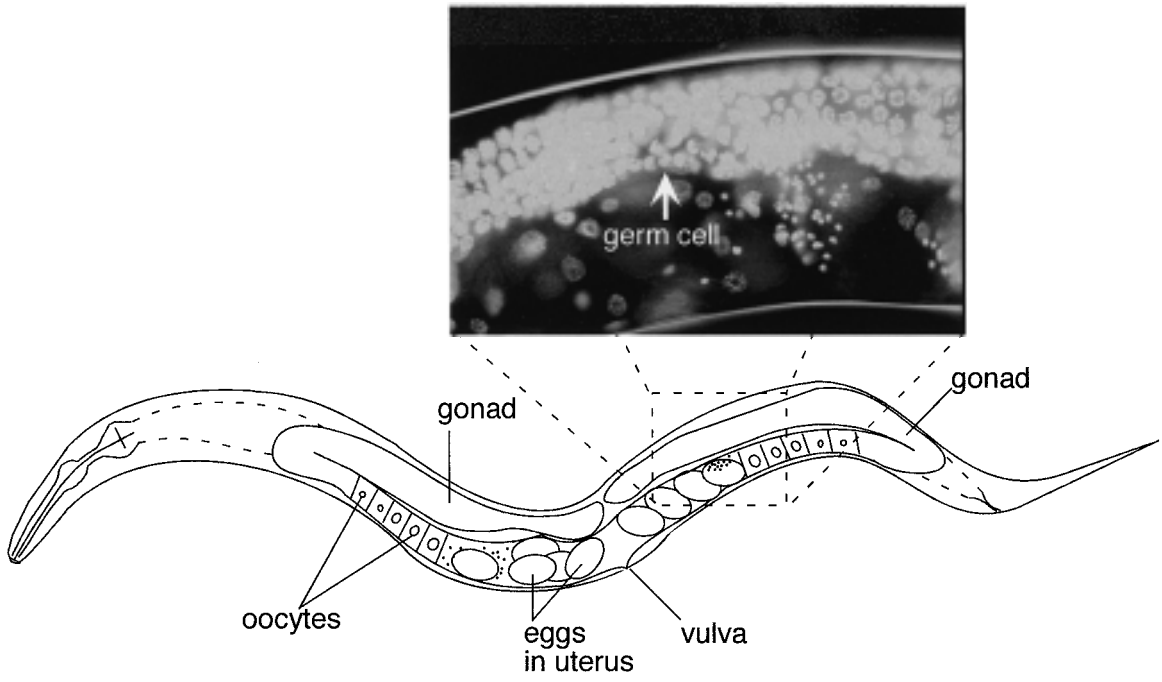

Fig. 1. Schematic diagram of the adult hermaphrodite *Caenorhabditis elegans* and DAPI-stained images of the germ cells.

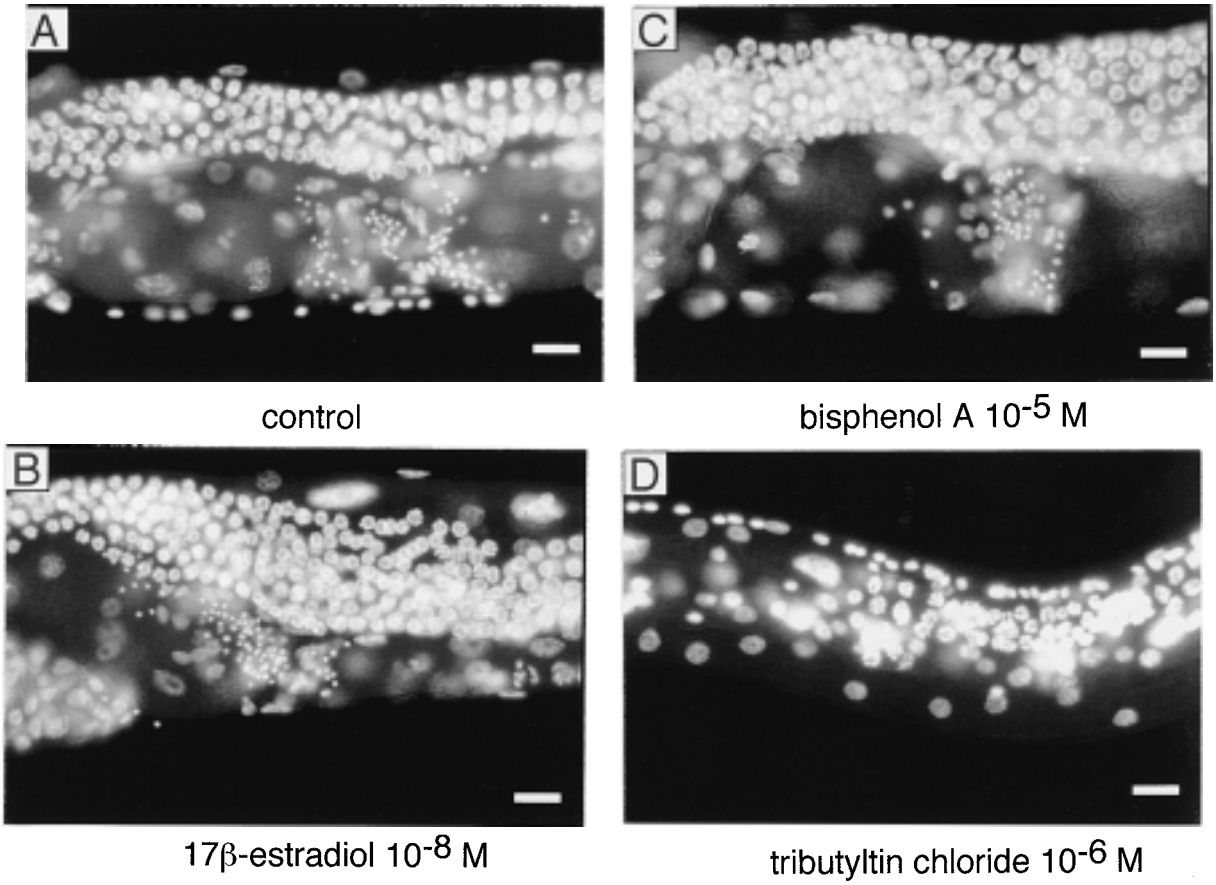

Fig. 2. DAPI-stained images of the germ cells in *Caenorhabditis elegans* exposed for 6 days to (A) 0.3 % ethanol (solvent control), (B) 10^{-8} M 17β-estradiol, (C) 10^{-5} M bisphenol A and (D) 10^{-6} M tributyltin chloride under fluorescence microscopy. Scale bar=10 μm.

The RPGs of E2, BPA and TBTCL-exposed worms are shown in Fig. 3. The mean ± S.D. of germ cells in control worms was 641 ± 132 (n=15). Significant increases in RPG were observed in E2 (10^{-10}–10^{-6} M) and BPA (10^{-9}–10^{-5} M)-exposed worms. The RPG was increased dose-dependently and maximal increases were observed in the worms exposed to 10^{-8} M E2 (156 ± 15.3% of control) and 10^{-5} M BPA (168 ± 20.0% of control). The RPG of TBTCL-treated worms showed a significant decrease at 10^{-9}–10^{-6} M. The minimal RPG was observed for 10^{-6} M TBTCL (30.2 ± 3.51% of control).

The lowest observed effective concentrations (LOEC) of E2, BPA and TBTCL were 10^{-10} M, 10^{-9} M and 10^{-9} M, respectively.

DISCUSSION

There are very few reports about short-term and sensitive *in vivo* methods of determining the effects of chemicals on reproductive organs.

The *C. elegans* assay used here to determine the effects of chemicals on one-generation exposure was inexpensive. NGM agar plates and the DAPI solution cost 42 cents/plate and 3 cents/m*l*, respectively. The tools required for this assay are widely available, such as a low temperature incubator and fluorescence microscope. To determine the one-generational effects of E2, BPA and TBTCL on germ cells required only 6 days with this assay. In the case of the uterotrophic assay using rats, it takes 10–12 weeks to prepare ovariectomized animals before treatment with chemicals [22]. DAPI-staining of the germ cells was quite simple and was accomplished within one hour. Moreover, we were able to count the stained germ cells without dissection, because *C. elegans* is almost transparent.

BPA is a known estrogen-mimetic that affects the reproductive organs of mammals, birds and fish [21, 24, 29]. As shown in Fig. 3, the RPG of *C. elegans* increased markedly after exposure to E2 and BPA. This finding indicates that BPA also exhibits estrogen-like effects on the reproductive organs of *C. elegans*.

It was reported that the LOEC of E2 for vitellogenin expression in cultured carp hepatocytes was 0.2×10^{-8} M [31]. The LOEC of E2 in the *C. elegans* assay was 10^{-10} M (Fig. 3A), suggesting a 20-fold higher sensitivity in the *C. elegans* assay. There are some reports of detecting E2 using vitellogenin in *C. elegans*. The levels of vitellogenin

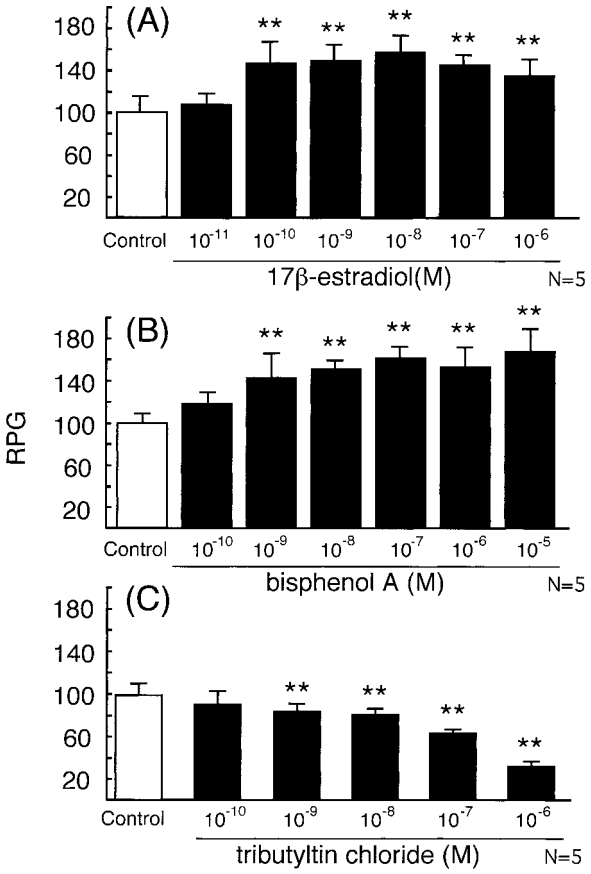

Fig. 3. The RPG (relative percentage of germ cells) of *Caenorhabditis elegans* exposed for 6 days to 17β-estradiol (A), bisphenol A (B) and tributyltin chloride (C). The RPG in each *C. elegans* was expressed by taking the mean value of the germ cells in control worms as 100%. RPG values are given as the mean ± S. D. of 5 worms. Data were analyzed by Dunnett's multiple comparison test to compare each treatment group with the control (* $p<0.05$; ** $p<0.01$).

mRNA and proteins in *C. elegans* have been reported to increase dose-dependently after exposure to E2 [9, 17]. These reports also support that *C. elegans* is a useful experimental animal for the screening of E2 and E2-like chemicals.

In the case of BPA exposure, the LOEC for vitellogenin expression in the cultured carp-hepatocyte assay was of 0.5×10^{-4} M [30]. This *C. elegans* assay showed a LOEC of 10^{-9} M (Fig. 3B). This suggests that the *C. elegans* assay has a 50,000-fold higher sensitive than the cultured carp-hepatocyte assay.

TBTCL (10^{-9} M) significantly decreased the RPG of *C. elegans* (Fig. 3C). To the best of the author's knowledge, TBT(CL) has no estrogen-like activity. It has been reported that a Gastropoda, *Hinia reticulata*, exhibits high sensitivity to TBT, showing imposex at 1.0 *n*g TBT as Sn/L (0.84×10^{-11} M) [34]. The LOEC of TBTCL in the *C. elegans* assay was 120-fold higher than the value for induction of imposex in *H. reticulata*.

Several reports have suggested that TBT inhibits the enzyme aromatase (CYP19) which catalyzes androgen to estrogen [14, 27]. Therefore, it has been theorized that TBT increases androgen levels through inhibition of aromatase activity and/or a suppression of androgen excretion. However, Cooke [8] reported that TBT did not inhibit aromatase activity at concentrations below 10^{-6} M. TBT was reported to decrease the activity of aromatase in the upper nanomolar range, the concentrations showing cytotoxicity [28]. Furthermore, Nakanishi *et al.* [25] reported that TBT increased aromatase activity at concentrations below 10^{-6} M. The mechanisms of the reproductive toxicity of TBT remain unclear.

The hormonal regulation of *C. elegans* may differ from that of vertebrates. However, *C. elegans* has steroid/thyroid hormone-receptor genes [18] and estrogen-binding proteins [15]. Furthermore, more than 83% of *C. elegans* gene-transcripts were found to have homologous gene-transcripts in humans [20]. Further investigation using *C. elegans* may provide additional insight into the toxicity of chemicals including environmental pollutants to the reproductive organs in humans at the molecular level.

In conclusion, the RPG determined by this assay is a novel and sensitive indicator of the effects of chemicals on reproductive organs, suggesting that the *C. elegans* assay is advantageous for studying the toxicity of environmental pollutants.

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REFERENCES

1. Ashby, J. and Tinwell, H. 1998. Uterotrophic activity of bisphenol A in the immature rat. *Environ. Health Perspect.* **106**: 719–720.
2. Austin, J. and Kimble, J. 1987. glp-1 is required in the germ line for regulation of the decision between mitosis and meiosis in *C. elegans*. *Cell* **51**: 589–599.
3. Bailey, S. K. and Davies, I. M. 1988. Tributyltin contamination in the Firth of Forth (1975–87). *Sci. Total Environ.* **76**: 185–192.
4. Brenner, S. 1974. The genetics of *Caenorhabditis elegans*. *Genetics* **77**: 71–94.
5. Brotons, J. A., Olea-Serrano, M. F., Villalobos, M., Pedraza, V. and Olea, N. 1995. Xenoestrogens released from lacquer coatings in food cans. *Environ. Health Perspect.* **103**: 608–612.
6. Capowski, E. E., Martin, P., Garvin, C. and Strome, S. 1991. Identification of grandchildless loci whose products are required for normal germ-line development in the nematode *Caenorhabditis elegans*. *Genetics* **129**: 1061–1072.
7. Celius, T., Haugen, T. B., Grotmol, T. and Walther, B. T. 1999. A sensitive zonagenetic assay for rapid *in vitro* assessment of estrogenic potency of xenobiotics and mycotoxins. *Environ. Health Perspect.* **107**: 63–68.
8. Cooke, G. M. 2002. Effect of organotins on human aromatase activity *in vitro*. *Toxicol. Lett.* **126**: 121–130.

9. Custodia, N., Won, S. J., Novillo, A., Wieland, M., Li, C. and Callard, I. P. 2001. *Caenorhabditis elegans* as an environmental monitor using DNA microarray analysis. *Ann. New York Acad. Sci.* **948**: 32–42.
10. Dodge, J. A., Glasebrook, A. L., Magee, D. E., Phillips, D. L., Sato, M., Short, L. L. and Bryant, H. U. 1996. Environmental estrogens: effects on cholesterol lowering and bone in the ovariectomized rat. *J. Steroid Biochem. Mol. Biol.* **59**: 155–161.
11. Emmons, S. W., Klass, M. R. and Hirsh, D. 1979. Analysis of the constancy of DNA sequences during development and evolution of the nematode *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U.S.A.* **76**: 1333–1337.
12. Gaido, K. W., Leonard, L. S., Lovell, S., Gould, J. C., Babai, D., Portier, C. J. and McDonnell, D. P. 1997. Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. *Toxicol. Appl. Pharmacol.* **143**: 205–212.
13. Gibbs, P. E., Bryan, G. W., Pascoe, P. L. and Burt, G. R. 1987. The use of the dog-whelk, *Nucella lapillus*, as an indicator of tributyltin (TBT) contamination. *J. Mar. Biol. Assoc. UK.* **67**: 507–523.
14. Heidrich, D. D., Steckelbroeck, S. and Klingmuller, D. 2001. Inhibition of human cytochrome P450 aromatase activity by butyltins. *Steroids* **66**: 763–769.
15. Hood, T. E., Calabrese, E. J. and Zuckerman, B. M. 2000. Detection of an estrogen receptor in two nematode species and inhibition of binding and development by environmental chemicals. *Ecotoxicol. Environ. Saf.* **47**: 74–81.
16. Kannan, K., Grove, R. A., Senthilkumar, K., Henny, C. J. and Giesy, J. P. 1999. Butyltin compounds in river otters (*Lutra canadensis*) from the northwestern United States. *Arch. Environ. Contam. Toxicol.* **36**: 462–468.
17. Kohara, S., Tominaga, N., Mitsui, Y., Takao, Y., Ishibashi, Y. and Arizono, K. 1999. Determination of a screening system of endocrine disruptors by induction of vitellogenin mRNA in *C. elegans* larvae. *J. Health Sci.* **45**: 37.
18. Kostrouch, Z., Kostrouchova, M. and Rall, J. E. 1995. Steroid/thyroid hormone receptor genes in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U.S.A.* **92**: 156–159.
19. Krishnan, A. V., Stathis, P., Permuth, S. F., Tokes, L. and Feldman, D. 1993. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* **132**: 2279–2286.
20. Lai, C. H., Chou, C. Y., Ch'ang, L. Y., Liu, C. S. and Lin, W. 2000. Identification of novel human genes evolutionarily conserved in *Caenorhabditis elegans* by comparative proteomics. *Genome Res.* **10**: 703–713.
21. Laws, S. C., Carey, S. A., Ferrell, J. M., Bodman, G. J. and Cooper, R. L. 2000. Estrogenic activity of octylphenol, nonylphenol, bisphenol A and methoxychlor in rats. *Toxicol. Sci.* **54**: 154–167.
22. Long, X., Steinmetz, R., Ben-Jonathan, N., Caperell-Grant, A., Young, P. C., Nephew, K. P. and Bigsby, R. M. 2000. Strain differences in vaginal responses to the xenoestrogen bisphenol A. *Environ. Health Perspect.* **108**: 243–247.
23. Lutz, I. and Kloas, W. 1999. Amphibians as a model to study endocrine disruptors: I. Environmental pollution and estrogen receptor binding. *Sci. Total Environ.* **225**: 49–57.
24. Matthews, J., Celius, T., Halgren, R. and Zacharewski, T. 2000. Differential estrogen receptor binding of estrogenic substances: a species comparison. *J. Steroid Biochem. Mol. Biol.* **74**: 223–234.
25. Nakanishi, T., Kohroki, J., Suzuki, S., Ishizaki, J., Hiromori, Y., Takasuga, S., Itoh, N., Watanabe, Y., Utoguchi, N. and Tanaka, K. 2002. Trialkyltin compounds enhance human CG secretion and aromatase activity in human placental choriocarcinoma cells. *J. Clin. Endocrinol. Metab.* **87**: 2830–2837.
26. Pedersen, S. N. and Lindholst, C. 1999. Quantification of the xenoestrogens 4-tert.-octylphenol and bisphenol A in water and in fish tissue based on microwave assisted extraction, solid-phase extraction and liquid chromatography-mass spectrometry. *J. Chromatogr. A* **864**: 17–24.
27. Saitoh, M., Yanase, T., Morinaga, H., Tanabe, M., Mu, Y. M., Nishi, Y., Nomura, M., Okabe, T., Goto, K., Takayanagi, R. and Nawata, H. 2001. Tributyltin or triphenyltin inhibits aromatase activity in the human granulosa-like tumor cell line KGN. *Biochem. Biophys. Res. Commun.* **289**: 198–204.
28. Sanderson, J. T., Boerma, J., Lansbergen, G. W. and van den Berg, M. 2002. Induction and inhibition of aromatase (CYP19) activity by various classes of pesticides in H295R human adrenocortical carcinoma cells. *Toxicol. Appl. Pharmacol.* **182**: 44–54.
29. Shilling, A. D. and Williams, D. E. 2000. Determining relative estrogenicity by quantifying vitellogenin induction in rainbow trout liver slices. *Toxicol. Appl. Pharmacol.* **164**: 330–335.
30. Smeets, J. M., Rankouhi, T. R., Nichols, K. M., Komen, H., Kaminski, N. E., Giesy, J. P. and van den Berg, M. 1999. *In vitro* vitellogenin production by carp (*Cyprinus carpio*) hepatocytes as a screening method for determining (anti)estrogenic activity of xenobiotics. *Toxicol. Appl. Pharmacol.* **157**: 68–76.
31. Smeets, J. M., van Holsteijn, I., Giesy, J. P., Seinen, W. and van den Berg, M. 1999. Estrogenic potencies of several environmental pollutants, as determined by vitellogenin induction in a carp hepatocyte assay. *Toxicol. Sci.* **50**: 206–213.
32. Smith, B. S. 1981. Male characteristics on female mud snails caused by antifouling bottom paints. *J. Appl. Toxicol.* **1**: 22–25.
33. Staples, C. A., Dorn, P. B., Klecka, G. M., O'Block, S. T., Branson, D. R. and Harris, L. R. 2000. Bisphenol A concentrations in receiving waters near US manufacturing and processing facilities. *Chemosphere* **40**: 521–525.
34. Stroben, E., Schulte-Oehlmann, U., Fioroni, P. and Oehlmann, J. 1995. A comparative method for easy assessment of coastal TBT pollution by the degree of imposex in Prosobranch species. *Haliotis* **24**: 1–12.
35. Takahashi, S., Tanabe, S., Takeuchi, I. and Miyazaki, N. 1999. Distribution and specific bioaccumulation of butyltin compounds in a marine ecosystem. *Arch. Environ. Contam. Toxicol.* **37**: 50–61.
36. Tsuda, T., Inoue, T., Kojima, M. and Aoki, S. 1995. Daily intakes of tributyltin and triphenyltin compounds from meals. *J. AOAC Int.* **78**: 941–943.
37. White, J. 1988. The anatomy, pp. 81–122. *In*: The Nematode *Carnorhabditis elegans* (Wood W. B. ed.), Cold Spring Harbor Laboratory, NY.